DEMONSTRATION OF CARDIAC GLYCOSIDE-LIKE SUBSTANCES IN BOVINE ADRENAL CORTEX AND PLASMA FROM HYPERTENSIVE AND NORMOTENSIVE SUBJECTS

by

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DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis are my own.

Karen Traill

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ABSTRACT

It has been postulated that essential hypertension develops and is maintained by a natriuretic hormone which has its effect through the inhibition of Na^+K^+ATPase. The work contained in this thesis measured two characteristics of this putative hormone; the ability to inhibit Na^+K^+ATPase and cross-reactivity with digoxin antibodies.

Two different assays were developed for the measurement of Na^+K^+ATPase activity and an extraction method was developed in which inhibitory substances were isolated from plasma according to their polarity, using Sep-Pak C_{18} cartridges. Extracts, prepared from plasma samples from normotensive and hypertensive subjects were measured for the capacity to inhibit Na^+K^+ATPase and to cross-react with digoxin antibodies. There was no significant difference between the two populations and there was no correlation between the inhibition and cross-reactivity or between blood pressure and either activity.

Dilutions of extracts from bovine serum and from bovine adrenal cortex cytosol produced dose-response curves for both inhibition and cross-reactivity. When the extracts were subjected to separation by high performance liquid chromatography (HPLC), these activities eluted in several fractions but inhibitory acivity and cross-reactivity co-eluted in only two positions.

The co-eluting fractions, obtained by HPLC separation of adrenal cortex cytosol, did not contain non-esterified fatty acids and could be steroid-like since they cross-reacted with digoxin, cortisol and corticosterone antibodies. Trypsin reduced the activity of these fractions by half and activity was not sensitive to heat (80^{o}C, 10 min). It was also shown that these fractions stimulated steroidogenesis in isolated adrenal zona glomerulosa and zona fasciculata cells.

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SECTION ONE

INTRODUCTION

1. HYPERTENSION

(a) Definition of hypertension

Epidemiological studies have shown that hypertension is associated with an increase in cardiovascular mortality and morbidity (Miall and Chinn, 1974; Dawber, 1980). The value at which the blood pressure of an individual is sufficiently raised for them to be described as hypertensive is an entirely arbitrary decision since, within a defined population, blood pressure is normally distributed (Hawthorne, et al, 1974). Guidelines set out by the World Health Organisation suggest values at which an individual should be considered hypertensive and consequently be treated with drugs to lower their blood pressure. However, these values do not take account of the increase in blood pressure with age which occurs in all but a few societies (Freis, 1976), so it has been suggested (Julius and Hansson, 1983) that the following values would be more suitable for clinical use: normotensive; <140/90 at age 17 to 40, <150/90 at age 41 to 60 and <160/90 at age >60, hypertensive; >160/95 at age 17 to 60 and >175/95 at age >60. Borderline hypertension is defined as a blood pressure between those of normotensive and hypertensive. These values apply to non-pregnant adults only because during normal pregnancy blood pressure decreases during the first two trimesters rising to values close to normal

towards the end of the third trimester (MacGillivray, et al, 1969). In children, blood pressure values have been reported to correlate better with height than age (Voors, et al, 1978).

During the 1960's, the inheritability of hypertension was the centre of a lively debate between Pickering and Platt, details of which can be found in Pickering's book: "High Blood Pressure" (Pickering, 1968a). Pickering thought that hypertension was due to many factors, inheritance being only one of them. Platt considered hypertension to be the result of a single dominant gene and cited the bimodal distribution of blood pressure within a population sample as evidence in support of this idea. Reports of blood pressure similarities between homozygous twins were also taken as evidence in favour of the single gene theory. However, the bimodal blood pressure distribution curves were found to be a statistical artefact and the now generally accepted view is that inheritance is only one of several predisposing factors.

It has been reported that as many as 15% of the population have high blood pressure (Hawthorne, et al, 1974) and in 95% of these cases no specific cause is found (Berglund, et al, 1976) so the hypertension is described as "essential". The term "essential" has historical roots (Pickering, 1968b), hypertension of this type is more correctly

described as "primary". The term "essential" was used because it was thought that the elevated blood pressure was necessary to maintain perfusion of organs, especially the kidney. In the remaining 5% of cases, it is possible to detect the cause of the raised blood pressure so the hypertension is described as secondary.

A list of the main causes of secondary hypertension is given in Table 1.1. Most commonly secondary hypertension is due to renal disease or renal vascular disease. Hypertension in renal vascular disease is caused by secondary hyperaldosteronism due to increased renin secretion. Primary hyperaldosteronism, or Conn's syndrome (Conn, 1955) is characterised by excess production of aldosterone by either an adenoma or bilateral micronodular hyperplasia. This condition is also characterised by a low plasma renin, low plasma potassium but no increase in ACTH secretion. The mineralocorticoid effects of glycyrrhetinic acid, which is present in liquorice, cause pseudo-hyperaldosteronism if liquorice is consumed in sufficient quantities.

Excessive production of adrenal hormones causes secondary hypertension in two other conditions; Cushing's syndrome or disease (Cushing, 1932) and phaeochromocytoma. In Cushing's disease the adrenal cortex produces increased amounts of hydrocortisone which in the majority of cases is

TABLE 1.1

MAIN CAUSES OF SECONDARY HYPERTENSION

Diseases of the kidneys or urinary tract

e.g. glomerulonephritis, chronic pyelonephritis, renal artery stenosis, renin secreting tumour.

Coarctation of the aorta

Phaeochromocytoma

Cushing's syndrome

Primary hyperaldosteronism (Conn's syndrome)

Pre-eclampsia

Acromegaly

CNS disorders

- increased intracranial pressure

Drug associated conditions

- oral contraceptive pill, glucocorticoids, carbenoxolone sodium, liquorice addiction

due to inappropriate ACTH secretion by the pituitary gland. Phaeochromocytoma is caused by a catecholamine-secreting tumour of chromaffin tissue, occurring at any site along the sympathetic chain, although 90% are located in the adrenal gland.

(b) Prognosis and treatment of hypertension

(i) Mortality and morbidity

For many decades it has been known that raised blood pressure is associated with increased mortality. Deaths as a result of extremely high blood pressure (systolic >180mmHg) are usually due to cerebral haemorrhage, dissecting aneurysms or heart failure. However, the majority of deaths due to hypertension are caused by coronary artery disease and occur at lower pressures (140-160mmHg systolic pressure) (Sleight, 1983).

Trials have shown that drug treatment of severe hypertension (diastolic 115-129mmHg) improves mortality and morbidity (Veterans Administration, 1967) and that systematic control of blood pressure can reduce all-cause mortality in subjects with diastolic pressures greater than 90mmHg (Hypertension Detection and Follow-up Program, 1979). Results from this latter trial suggested that mild hypertensives would benefit from treatment. Analysis of the results obtained for mild hypertensives (diastolic

pressure 91-104mmHg) in the Veterans Administration trial indicated that effectiveness of treatment depended on the initial blood pressure and that treatment had the greatest effect in patients with cardiovascular disease or who were over 50 years old (Veterans Administration, 1972). The value of treatment to the remaining patients was thought to be questionable considering the side-effects of the antihypertensive drugs. The Veterans Administration trials were based on an extremely compliant group of patients. Several recent studies on the treatment of mild hypertension have been based on normal populations (MRC Working Party, 1985; Australian National Blood Pressure Study, 1980; Helgeland, 1980). All the studies concluded that the incidence of cerebrovascular events was reduced in treated subjects but there was disparity in the conclusions about cardiovascular mortality. The MRC trial (1985) reported a decreased incidence of all cardiovascular events but no difference in the all-cause mortality rate. This is in contrast with the Australian trial (1980) where there was a decrease in mortality in the treated group with a two thirds reduction in deaths due to cardiovascular disease. The Oslo study (Helgeland, 1980) showed no difference in cardiovascular morbidity between the treated and untreated groups. The different conclusions reached in these trials may be due to differences in patient selection and sample size. The Australian trial comprised 3427 subjects, the

MRC trial 17354 subjects, whereas the Oslo study assessed 785 subjects.

(ii) Treatment of Hypertension

There are a large number of drugs available for the treatment of hypertension (Table 1.2) which produce their effect by a variety of different mechanisms many of which are not clearly understood. Many of the early drugs are no longer used for the chronic treatment of hypertension because of their undesirable and, in some instances, life-threatening side effects. This is particularly true of ganglion blockers and adrenergic neurone blocking agents. Centrally acting drugs such as α-methyldopa and clonidine are effective blood pressure lowering agents but their usefulness is limited by their sedative effect. Direct vasodilation was an attractive method for reducing blood pressure but the drugs that have been developed have many side effects which again limit their use. Vasodilators, e.g. hydralazine, also need to be used in conjunction with a diuretic and a β-blocker to compensate for the fluid retention and the increased sympathetic discharge.

The drugs most commonly used to treat hypertension are diuretics and β-blockers but they too have a number of metabolic side effects which were recently highlighted in the MRC trial on treatment of mild hypertension (MRC Working Party, 1981).

TABLE 1.2

DRUGS AVAILABLE FOR THE TREATMENT OF HYPERTENSION

Type of drug	Example	Side Effects
Adreneric neurone blockers	guanethidine	postural and exercise hypotension
α-adrenergic receptor blockers	prazosin	sedation dizziness
Centrally acting drugs	α-methyldopa	sedation fluid retention
Direct vasodilators	hydralazine	tachycardia fluid retention
Diuretics	bendrofluazide	hypokalaemia hyponatraemia hyperuricaemia hyperglycaemia hyperlipidaemia
β-adrenergic receptor blockers	metoprolol	bradycardia heart failure bronchospasm
Angiotensin-converting enzyme inhibitors	captopril	rash hyperkalaemia proteinuria agranulocytosis
Calcium antagonists	nifedipine	flushing oedema tachycardia

More recent additions to the list of drugs available are the angiotensin converting enzyme inhibitors; captopril and enalapril, and calcium channel blockers; e.g. nifedipine, verapamil. Initially high doses of captopril were used to treat hypertension and serious side effects were reported. However, lower doses were found to be capable of controlling blood pressure, thus reducing the incidence and severity of the side effects. Calcium antagonists, as they are known, inhibit the inward passage of calcium ions across myocardial and vascular smooth muscle cell membranes.

All of the drugs have undesirable side effects to a greater or lesser degree and it is the balance between the possible benefits of treating moderately raised blood pressure and the side effects of the treatment which is currently under debate. Although antihypertensive drugs reduce blood pressure they do not produce their effect by treating the cause of the hypertension, with the possible exception of the calcium blockers. It will not be possible to produce drugs which specifically treat the cause(s) of essential hypertension until it is known how essential hypertension develops and is maintained.

2. SALT AND HYPERTENSION

The reduction of salt intake in hypertensives as a method

of reducing blood pressure has been a subject of great interest especially since there are a large number of borderline hypertensives in whom the benefits of drug treatment may be outweighed by the side-effects of the drugs. Prior to the development of anti-hypertensive drugs it was possible to treat severe hypertension by salt restriction. It was shown that by drastic reduction of the sodium intake to 5-10mmol/day it was possible to lower the blood pressure in patients suffering from hypertension (Kempner, 1948). Unfortunately this diet of rice and fruit is notorious for being extremely unpalatable and so it is difficult for patients to comply. When the salt intake was increased to 25-35mmol/day the blood pressure rose but not to the pretreatment levels (Watkin, et al, 1950). Kawasaki, et al, (1978) have suggested that there is a sub-group of essential hypertensives who are salt-sensitive in an analogous way to the Dahl salt-sensitive rats. They found that some patients responded to a low sodium diet (9mmol/day) by a significant reduction in blood pressure while others did not. The salt-sensitive group had higher blood pressures when on an average (100mmol/day) and high (240mmol/day) sodium diet. No difference in blood pressure was observed between the average and high sodium diets and it should be noted that the sodium content of the "average Western" diet lies between the values chosen by Kawasaki, et al, as average and high.

Studies on moderate dietary salt reduction in subjects with hypertension have shown it has a small (approx. 7mmHg) but statistically significant effect on blood pressure (MacGregor, et al, 1982; Morgan, et al, 1978). Although anti-hypertensive drugs resulted in a greater reduction in blood pressure, the decrease due to moderate salt restriction was sufficient in many patients to reduce the blood pressure to within the normal range (Morgan, et al, 1978). It has been pointed out (Laragh and Pecker, 1983) that essential hypertension is not a homogeneous entity and that salt restriction could be of therapeutic use in selected patients e.g. low renin hypertensives, but would not be universally effective. This is in agreement with the suggestion of Kawasaki, et al, (1978) that there is a sub-group of essential hypertensives who are salt-sensitive.

The importance of salt intake in the development of hypertension has been a subject of great debate (Proc. Int. Soc. Hypertension, 1979). It is largely agreed that extremely high salt intakes are associated with a high incidence of hypertension. For example, in populations such as that found in north-east Japan, where the daily consumption of salt was around 400mmol, blood pressure and death from apoplexy was higher than in other regions of Japan (Sasaki, 1964). Death due to apoplexy was assumed to reflect death as a result of intracranial haemorrhage, although apoplexy can also be due to intracranial

thrombosis. The daily salt intake of the average "Western" diet is 150-200mmol and the current debate is whether the population at large would benefit from a moderate reduction in salt intake.

In "Western" societies hypertension is relatively common and blood pressure increases with age. Studies of "primitive" societies have shown that salt intake is much lower, the incidence of hypertension is low or absent and blood pressure does not increase with age (Freis, 1976). It has been proposed that the difference in incidence of hypertension between these two types of society is due to the increased salt intake in the "Western" societies since "primitive" societies which consume large quantities of salt also develop high blood pressure. These observations on the "primitive" societies are at the centre of the debate about salt and hypertension. Simpson (1979) is one author who has pointed out that there are other differences between the societies, including the overall composition of the diet, which could be responsible for the different levels of blood pressure.

The diet of "primitive" populations is not only low in sodium but also high in potassium. It has been suggested that the lack of hypertension in these societies is due to the increased potasssium intake. The effects of dietary potassium on blood pressure are complicated (Tannen, 1983);

potassium depletion lowers blood pressure in normotensive and hypertensive animals but a high potassium intake has no effect on normotensives but lowers blood pressure in hypertensives. However, a recent large population study reported that there was a negative correlation between urinary potassium excretion and blood pressure (Intersalt Cooperative Research Group, 1988). Another cation which has been implicated in the development of hypertension is calcium (McCarron, 1985), the importance of this cation has been debated (MacGregor, 1985).

Other dietary alterations which have been shown to have blood pressure lowering effect include a vegetarian diet (Rouse, et al, 1983; Margetts, et al, 1986) and supplementation of the diet with polyunsaturated fats (Puska, et al,1983) or linoleic acid (Comberg, et al, 1978). High alcohol consumption (Saunders, 1987) and obesity (Chiang, et al, 1969) are also associated with hypertension and a recent study indicated that these variables were more strongly related to blood pressure than urinary sodium excretion (Intersalt Cooperative Study Group, 1988).

3. HYPOTHESISED CAUSES OF ESSENTIAL HYPERTENSION

Many different theories have been proposed to explain how essential hypertension develops and is maintained. The

physiology of blood pressure control is complex depending on the inter-relationship of several different endocrine and nervous systems. As discussed below, abnormal functioning of normal control systems could be responsible for the increased vasoconstriction observed in essential hypertension which is the only universal difference between normotensives and essential hypertensives. It is now generally accepted that essential hypertension is a multifactorial condition and as such it is likely that there are several different causes or a combination of predisposing features.

The heterogeneous nature of essential hypertension should not preclude the investigation of these subjects for the presence of a single factor which may be present only in a sub-group which is characterised by a common defect e.g. inability to deal effectively with a salt load. Within the group of patients described as essential hypertensives it is likely that there are several sub-groups whose hypertension may arise by the mechanisms described in the theories outlined below.

Brown and Macquin (1981) suggested that intermittent raised secretion of adrenaline from the adrenal medulla accumulates in sympathetic nerve endings and when released acts at pre-synaptic β_2-receptors thus increasing noradrenaline release. This in turn stimulates the

activity of the sympathetic nervous system leading to increased vasoconstriction and blood pressure. 78 studies of plasma levels of catecholamines in normotensives and hypertensives were surveyed and, although most studies reported increased levels in hypertensives, the difference between the two populations was significant in only 40% of the studies (Goldstein, 1983). Goldstein noted the problems encountered when trying to measure catecholamines; careful collection of blood samples was essential in order to avoid oxidation of catecholamines and the avoidance of stress, caffeine and high salt was also important. Goldstein also reported that when total catecholamines were measured hypertensives always had significantly higher levels but that noradrenaline and adrenaline were raised independently of one another. It would be expected from Brown and Macquin's theory that levels of both catecholamines would be increased in parallel.

An alternative hypothesis suggests that idiopathic hyperaldosteronism (IHA) is actually an extreme variant of essential hypertension (EHT) and not a variant of Conn's syndrome (Padfield, et al, 1981). Low renin is a feature of Conn's syndrome but is also present in 25% of essential hypertensives (Dunn and Tannen, 1974). Electrolyte abnormalities and angiotensin II (AII) responsiveness were more similar to EHT than Conn's syndrome and Padfield, et al, suggest that IHA is in fact a subset of EHT and that

there is a wider than normal range of aldosterone concentrations in EHT.

Since infusion of prostaglandins (PGE_2 and PGI_2), into the renal artery, have been shown to decrease blood pressure (Baer and McGiff, 1979) and cause natriuresis (Jones, et al, 1981) it is possible that hypertension could result from inadequate renal production of these prostaglandins. Decreased production of PGE_2 has been reported in both primary and secondary hypertension (Sato, et al, 1983) but Rathaus, et al (1983) reported decreased production in low renin EHT but not in normal renin EHT. At present, it is not clear whether lower levels of natriuretic prostaglandins are responsible for, or a consequence of, hypertension.

The failure to mobilise renal dopamine in response to a saline load was observed in essential hypertensives (Perkins, et al, 1980) and, since dopamine is a potent natriuretic agent (Lee, 1982), it has been suggested (Lee, 1981) that this could be a cause of essential hypertension. In the natriuretic hormone hypothesis (de Wardener and MacGregor, 1980), it is postulated that essential hypertension develops and is maintained by an entirely novel compound which acts as an endogenous Na^+K^+ATPase inhibitor. The stimulus for release of this inhibitor is central volume expansion which is a consequence of a

primary renal defect characterised by failure to cope with a salt load. The primary renal fault could be failure to mobilise renal dopamine.

In 1981, de Bold, et al, reported that intravenous injection of rat atrial homogenate, into control rats, produced both a natriuresis and diuresis. This spawned a field of research which advanced at such a pace that by 1984 the substances responsible, the atrial natriuretic peptides, had been purified, sequenced and the gene cloned in both rat (Yamanaka,et al, 1984) and man (Oikawa,et al, 1984). The physiological role of the atrial natriuretic peptides has yet to be elucidated but they are likely to have an important role in the fluid balance of the body. Extensive reviews can be found in the following sources; Genest, 1986, Needleman and Greenwald, 1986, Maack, et al, 1985. It has been apparent for some time that the natriuretic peptides were not the putative natriuretic hormone of de Wardener and MacGregor (1980) since it was shown that they did not inhibit Na^+K^+ATPase (Thibault, et al, 1983).

4. THE NATRIURETIC HORMONE

In this thesis the natriuretic hormone hypothesis was investigated by examining plasma and serum for the presence of endogenous Na^+K^+ATPase inhibitors. Work which led to

the development of the hypothesis, the investigation of the hypothesis and related work were extensively reviewed recently by de Wardener and Clarkson (1985). Since work on endogenous Na^+K^+ATPase inhibitors has advanced in parallel with work in this thesis, this section will only review literature which had been published before the commencement of this thesis in order to present an idea of the state of the field at the outset of this work.

(a) Involvement of a humoral factor in the natriuresis of volume expansion.

Originally essential hypertension was thought to be associated with volume expansion and consequently a large amount of effort was directed towards the investigation of the natriuresis of extracellular fluid volume expansion. In 1961, de Wardener, et al, demonstrated that a humoral factor was responsible for the natriuresis following volume expansion in the dog. When two dogs were cross-circulated (parabiosed), volume expansion of one dog resulted in natriuresis in both dogs, although the natriuresis in the recipient dog was 3 to 10 times less. Pearce, et al, (1969) showed that the natriuretic response to volume expansion was exaggerated in DOCA-salt treated rats and depressed in salt deficient rats. When these rats were cross-circulated the response of the salt deficient rats was restored without altering the response of the DOCA-salt

rats which indicated that a humoral factor, produced by the DOCA-salt rat, was responsible for the natriuretic response. Another hypertensive rat model, the Dahl salt-sensitive rat, develops hypertension when fed a high salt diet. Dahl, et al, (1967) parabiosed salt-sensitive and salt-resistant rats and noted that the blood pressure of both rats increased when fed on a high salt diet. It was suggested that the hypertension developed in the salt-resistant rat as a consequence of a humoral factor produced by the salt-sensitive rat.

The onset of natriuresis of volume expansion was examined by Pearce, et al, (1974) using cross-circulated rats where one rat was volume expanded using whole blood. The natriuresis was of gradual onset taking 60 to 80 minutes to reach a peak. Since infusing the donor rat for one hour before cross-circulating with the recipient did not affect the rate of onset of natriuresis, the natriuretic effect of volume expansion was not due to substances present in the infused blood. No significant natriuresis was observed if the donor was not expanded which suggested that an endogenous substance was responsible.

Extracts have been prepared from the plasma of volume expanded subjects in an attempt to isolate the humoral factor(s) responsible for the natriuresis. Extracts from the plasma of volume expanded cows (Lichardus, et al,

1968), dogs (Buckalew and Nelson, 1974) and rats (Pearce and Veress, 1974) have been demonstrated to be natriuretic in assay rats. The natriuretic activity present in volume expanded rat plasma was fractionated on Sephadex G-50 (Pearce and Veress, 1974) and it was observed that only a large molecular weight fraction from the volume expanded rat plasma had a greater natriuretic capacity than the control rat.

Central volume expansion of human volunteers, by water immersion up to the neck, resulted in a significant natriuresis and an increase in the natriuretic fraction extracted from urine (Epstein, et al, 1978).

It was hypothesised that volume expanded hypertension was due to a factor which inhibited Na^+K^+ATPase (Haddy and Overbeck, 1976) so the plasma of volume expanded experimental animals has been investigated for the presence of Na^+K^+ATPase inhibitory capacity and it has been shown that chromatographic separation produces several active fractions. Gonick, et al, (1977) found six peaks of inhibitory activity on gel filtration of plasma on Sephadex G-25 but only one peak contained significantly greater activity than the control plasma. HPLC separation of plasma on cation exchange and reverse-phase octadecylsilane columns revealed two peaks capable of both Na^+K^+ATPase

inhibition and cross-reacting with digoxin antibodies (Gruber, et al, 1980). These two peaks were present in increased amounts in volume expanded dogs.

Plasma extracts from volume expanded rats, prepared by HPLC separation, exhibited antinatriferic activity (Bealer, et al, 1983). Antinatriferic activity is defined as the inhibition of sodium transport across anuran membranes, assessed by a decrease in the short circuit current. Buckalew and Nelson (1974) reported that ultrafiltrates of plasma from volume expanded dogs had a greater capacity to inhibit the toad bladder short circuit current than that of volume depleted dogs. However, although Buckalew (1975) was able to repeat these findings Brown, et al, (1974), using the same ultrafiltration method, were not.

(b) Effect of sodium intake on Na^+K^+ATPase inhibitor levels

The effect of altering sodium levels on the circulating concentration of endogenous Na^+K^+ATPase inhibitors has been investigated using a cytochemical method to measure inhibition. In this method inhibition of Na^+K^+ATPase is measured as a stimulation of glucose-6-phosphate dehydrogenase in tissue slices as assessed using densitometric measurement of the colour produced by the reaction of the dehydrogenase with a dye (Fenton, et al, 1982). Using this assay, de Wardener, et al, (1981) and

MacGregor, et al, (1981) showed that the ability of plasma to stimulate glucose-6-phosphate dehydrogenase increased with an increase in sodium intake. The glucose-6-phosphate dehydrogenase stimulating capacity of a hypothalamic extract has also been shown to increase when rats are fed a high salt diet (Alaghband-Zadeh, et al, 1983).

(c) Demonstration of an inherited kidney defect in genetically hypertensive rats.

Results from kidney cross-transplantation experiments in genetically hypertensive rats have demonstrated that the kidneys are involved in the development of hypertension in these rats.

Bianchi, et al, (1974) selectively bred a rat model of essential hypertension which was normotensive at birth but which became hypertensive as it matured. These rats were described as spontaneously hypertensive rats (SHR). When kidneys were transplanted between SHR and normotensive rats the recipients of the kidneys from SHR rats developed hypertension irrespective of the original strain and the recipients of the kidneys from the normotensive rats remained or became normotensive (Bianchi, et al, 1974). This demonstrated that hypertension could develop in a normal rat if it was the recipient of a genetically

defective kidney. These experiments were performed on adult rats who were already hypertensive. When the cross-transplants were carried out on immature rats, prior to the development of hypertension in the SHR, the same results were obtained indicating that the development of hypertension in these rats was due to a genetic defect in the kidney and not due to kidney damage as a result of the raised blood pressure.

Dahl, et al, (1962) selectively bred two strains of rat which were described as hypertension-resistant and hypertension-prone. The hypertension-prone rats became hypertensive on a high salt diet and are often referred to as salt-sensitive, the hypertension-resistant rats being described as salt-resistant. It was observed that, when kidneys were cross-transplanted between the strains, the recipients of the kidneys from the hypertension-prone rats became hypertensive irrespective of the recipient's strain (Dahl and Heine, 1975). Transplantation of a kidney from a hypertension-resistant rat resulted in a blood pressure similar to that of control rats.

(d) Source of circulating inhibitor

Kaloyanides, et al, (1977) performed organ ablation experiments and found that the natriuretic response to volume expansion was lost by decapitation but not by

thyroparathyroidectomy, hypophysectomy or adrenalectomy leading them to suggest that the natriuretic factor was produced in the brain. The anteroventral third ventricle (AV3V) of the rat brain is critical for normal fluid-electrolyte regulation and cardiovascular homeostasis. It has been shown that the natriuretic response of AV3V-lesioned rats to volume expansion with saline is attenuated when compared with control rats (Bealer, et al, 1983) and that AV3V-lesioned rats no longer respond to DOCA-salt treatment with an increase in blood pressure (Songu-Mize, et al, 1982). When different tissues of the rat were analysed for the presence of substances capable of stimulating glucose-6-phosphate dehydrogenase only the hypothalamic extract contained any activity (Alaghband-Zadeh, et al, 1983). These results suggest that the source of the circulating inhibitor could be the AV3V region of the brain, specifically the hypothalamus. However, it has been reported that digoxin-like immunoreactivity was present in the adrenal gland of rat (Schreiber, et al, 1981a) and of the rabbit (Schreiber, et al, 1981b). This immunoreactivity could be due to cross-reactivity of known adrenal steroids with antibodies to digoxin. If further investigation demonstrated that the adrenal gland contained a factor, or factors, which was capable of inhibiting Na^+K^+ATPase, as well as cross-reacting with digoxin antibodies, a novel factor produced in the adrenal gland, and possibly under hypothalamic

control, could be responsible for the immunoreactivity which has been measured.

(e) Extraction and purification of cardiac glycoside-like substances

Several different sources have been investigated for the presence of endogenous cardiac glycoside-like activity. Measurement of activity has employed one or several of the following assays; inhibition of Na^+K^+ATPase, antinatriferic activity, cross-reactivity with antibodies to cardiac glycosides, displacement of tritiated ouabain from its binding site on erythrocytes, inhibition of ion fluxes from blood cells.

When plasma, urine or tissue are subjected to extraction and purification methods the production of artefacts becomes a serious consideration. All starting materials have the capacity to produce artefacts, whether it is body fluids or tissue, due to the presence of e.g. ions, hormones, fatty acids, which, if sufficiently concentrated, will interfere in assays for cardiac glycosides. For this reason it is necessary to use as many assays as possible, each depending on different properties of the factor. In practice the quantity of the extract and, to a lesser extent, financial and time constraints determine the number of assays used to test the extract. Problems with

artefacts can also be reduced by attempting to specifically remove substances during the isolation procedure which are known to interfere in assays. The use of cultured cells has been advocated as a possible way of avoiding the contamination of starting material with undesirable substances and avoiding problems associated with artefacts arising from the effects of extraction techniques on normal plasma or tissue constituents (Mir, et al, 1987). The extent of the problem with artefacts varies depending on the nature of the starting material, the concentration factor, the extraction method and the assays used.

(i) Plasma

Chromatographic separation of plasma and serum has demonstrated that several active fractions are present. HPLC separation of plasma from volume expanded dogs produced two peaks of co-eluting Na^+K^+ATPase inhibition and digoxin cross-reactivity which were present in higher concentrations than in the plasma of hydropenic dogs (Gruber, et al, 1980). Extracts prepared by HPLC of volume expanded rat plasma contained antinatriferic activity in control rats but not in AV3V-lesioned rats (Bealer, et al, 1983). Anion exchange chromatography of plasma produced three peaks capable of Na^+K^+ATPase inhibition and inhibition of [^{3}H]-ouabain binding in nearly half of the normotensives and all of the hypertensives studied (Cloix, et al, 1983).

(ii) Urine

A urine extract prepared by gel filtration on Sephadex G-25 was natriuretic (Bougoignie, et al, 1974), antinatriferic (Kaplan, et al, 1974) and capable of inhibiting sodium transport in the isolated rabbit collecting tubule from the peritubular but not from the luminal surface (Fine, et al, 1976). Only the extract prepared from the urine of uraemic patients was endowed with these characteristics. A similar extract was prepared by Poston, et al, (1982). Normotensive volunteers were given 9α-fluorocortisol until they underwent natriuresis and escaped from the salt retaining effect of the mineralocorticoid; so called mineralocorticoid escape. Post-escape urine extract from these volunteers significantly inhibited the ouabain-sensitive sodium efflux rate from leucocytes but pre-escape urine did not.

(iii) Tissue extracts

Endogenous cardiac glycoside-like activity has been reported in extracts from guinea pig brain (Fishman, 1979), rat brain (Lichtstein and Samuelov, 1980), bovine hypothalamus (Haupert and Sancho, 1979) and rat hypothalamus (Alaghband-Zadeh, et al, 1983). Each of the investigations used different assays for the assessment of activity but all used acetone extraction which has been demonstrated to produce artefacts (Whitmer, et al, 1982).

It has also been reported that acetone-HCl extraction of guinea pig heart produced non-specific irreversible inhibitors of Na^+K^+ATPase but that aqueous extraction produced a specific inhibitor which was also capable of inhibiting [^{3}H]-ouabain binding (De Pover, et al, 1982).

Digoxin-like immunoreactivity was found in extracts from rat adrenal glands by Schreiber, et al, (1981a) using acetone and organic solvent extraction. Thin layer chromatography separation showed that several fractions were responsible for the immunoreactivity.

Using gel filtration on Sephadex G-25 followed by polyacrylamide gel electrophoresis, Raghaven and Gonick (1980) extracted a fraction from the kidneys of volume expanded rats which was antinatriferic and would inhibit Na^+K^+ATPase. The rationale for this approach was that if volume expansion increased the concentration of the inhibitor then more would be bound to the receptors in the kidney so the kidney could provide an enriched source.

(iv) Amphibian plasma and skin

Since the poison glands in the skin of the toad, Bufo marinus, contain a cardiac glycoside-like substance, Flier (1978) prepared extracts from the skin of Bufo marinus and two other species of toad. Although the other two species of toad did not have poison glands on their skin, extracts

from all three species contained comparable amounts of activity which was measured as inhibition of Na^+K^+ATPase, inhibition of $^{42}K^+$influx into erythrocytes and inhibition of [^{3}H]-ouabain binding. Purification of skin extracts from four species of toad identified the active substances in two species; Bufo and Atelopus, as bufodienolides and in the other two species; Dendrophyryniscus and Melanophryniscus, the active substances were more polar and of unknown structure (Flier, et al, 1980). In the same paper, skin extracts from over thirty species of frog were reported to contain low levels of ouabain-like compounds. Flier, et al, (1980) suggest that the ouabain-like substances may be present not only as a means of defence but also as physiological regulators of Na^+K^+ATPase since amphibian skin is important for sodium and water homeostasis in these species and the skin is rich in the enzyme. Plasma from the toad, Bufo marinus, was shown to contain substances which inhibited the binding of [^{3}H]-ouabain to human erythrocytes (Flier, et al, 1979) but both plasma and skin from the frog, Rana pipiens, lacked activity. Toad plasma was also capable of cross-reacting in a digoxin RIA and preincubation of the toad plasma with anti-digoxin or anti-ouabain antibodies prevented the plasma from inhibiting [^{3}H]-ouabain binding to erythrocytes. Since Bufonid erythrocytes were incapable of binding ouabain, the toad would be expected to be immune to the effects of this endogenous ouabain-like compound.

(f) Involvement of endogenous Na^+K^+ATPase inhibition in uraemia

Cardiac glycoside-like activity has been reported in plasma and urine of patients with uraemia, a condition which is often associated with expansion of the extracellular fluid volume.

It has been known for some time that there are ion transport abnormalities associated with uraemia. These have been measured as a decrease in ouabain-sensitive Na^+K^+ATPase activity in erythrocyte membranes (Cole, 1973) and a decrease in sodium efflux from leucocytes (Edmondson, et al, 1975a). It has also been reported that there is a decrease in Na^+K^+ATPase activity in intestine (Kramer, et al, 1974) and in brain (Minkoff, et al, 1972) in experimental uraemia. A humoral factor was implicated when it was reported that the sodium efflux of control erythrocytes decreased when they were incubated in uraemic serum (Kramer, et al, 1976) and that there was significant inhibition of normal brain Na^+K^+ATPase by uraemic serum (Minkoff, et al, 1972). It was subsequently reported that the results for digoxin levels in two out of seven patients with renal failure receiving digoxin could vary from 0.3-3ng/ml depending on which digoxin kit was used to measure the values (Belpaire, 1975). Similar results were reported by Graves, et al, (1983) who measured digoxin

levels in patients with renal failure who were not receiving digoxin and noted that more than 60% had false positive values in most assays. Samples showing cross-reactivity were not tested for the ability to inhibit Na^+K^+ATPase.

(g) Involvement of endogenous Na^+K^+ATPase inhibition in hypertension

Sodium transport in hypertension has been studied in intact cells and using extracts of plasma and urine.

Comparing normotensive and hypertensive dogs, Overbeck, et al, (1976) observed that the ouabain-sensitive ^{86}Rb uptake of small mesenteric arteries and splanchnic veins from hypertensive dogs was depressed when they were incubated in the dogs own plasma. Since ouabain-sensitive ^{86}Rb uptake is considered to be a direct function of the transport activity of Na^+K^+ATPase, this suggests that the enzyme is inhibited in hypertensive dogs.

The sodium content of leucocytes has been shown to be higher in hypertensive subjects and was associated with a decreased rate constant for sodium efflux (Edmondson, et al, 1975b). Poston, et al, (1981) observed that sodium transport was impaired in both erythrocytes and leucocytes from hypertensives. A humoral factor would appear to be

responsible for this since incubation of leucocytes from normotensives in serum from hypertensives resulted in sodium transport which was impaired to the same degree as hypertensives (Poston, et al, 1981).

A raised internal sodium concentration has also been observed in lymphocytes from hypertensives and from the normotensive relatives of hypertensives (Ambrosioni, et al, 1981). In the essential hypertensives the lymphocytic sodium concentration significantly correlated with systolic, diastolic and mean blood pressure. There was no such correlation with blood pressure in the normotensives.

Ouabain-sensitive sodium efflux rates in leucocytes were also reported to be lower in normotensive subjects who had one or more first degree relative with essential hypertension than in normotensive subjects with no such family history (Heagerty, et al, 1982). It was suggested that, since blood pressure was normal in these subjects, a reduction in ouabain-sensitive sodium efflux was not directly involved in hypertension. Subsequent editions of the Lancet contained letters arguing against this interpretation and replies from Heagerty, et al, which demonstrate the lack of agreement on this point which prevailed in this field at the outset of this work. It was argued that a primary genetic abnormality, measured as a decrease in sodium efflux, may not produce hypertension

for many years or may require an environmental trigger e.g. salt intake. Other correspondents pointed out that there was a body of literature which indicated that a humoral factor was responsible for the reduced Na^+K^+ATPase activity.

Hamlyn, et al, (1982) found that the ability of deproteinised plasma to inhibit Na^+K^+ATPase was correlated with mean arterial pressure in the hypertensives but not in the normotensives. However, when the normotensives were included in the statistics a greater correlation coefficient was obtained. Hamlyn, et al, (1982) also measured the cross-reactivity of their plasma extracts with digoxin antibodies and found no correlation between the amount of inhibition and the amount of cross-reactivity. However, Gruber, et al, (1982) observed a correlation between digoxin cross-reactivity and systolic and diastolic blood pressure in experimentally induced hypertension in monkeys.

It has also been reported that injection of digoxin antibodies into DOCA-salt hypertensive rats lowers their blood pressure (Kojima, et al, 1982).

(h) The natriuretic hormone hypothesis.

Results from parabiosis experiments between salt-sensitive

and salt-resistant rats led Dahl, et al, (1967) to suggest that a humoral factor was responsible for the sustained rise in blood pressure in the salt-sensitive rats fed a high salt diet. This hypothesis was extended by de Wardener and MacGregor (1982) to explain how a natriuretic hormone, which acts by inhibition of Na^+K^+ATPase, could be responsible for the development and maintenance of essential hypertension.

In the natriuretic hormone hypothesis it is postulated that, in essential hypertension, there is an inherited kidney defect which is characterised by an inability to excrete sodium. The accumulation of sodium and water which results from this produces an increase in the intrathoracic blood volume which stimulates the release of a Na^+K^+ATPase inhibitor, possibly from the hypothalamus. The Na^+K^+ATPase inhibitor acts on the nephron to produce natriuresis but also acts on peripheral blood vessels resulting in vasoconstriction and so a central increase in blood volume which in turn stimulates further release of the natriuretic hormone. Inhibition of the Na^+K^+ATPase results in an increase in sodium concentration inside the cell so there is no electrochemical gradient to drive the sodium-calcium exchange (Blaustein and Hamlyn, 1983). Increased intracellular calcium would lead to increased muscle contraction and so to increased vascular resistance. Cardiac glycosides have been shown to increase sensitivity

of smooth muscle to vasoconstrictive agents (Brender, et al, 1970) which is another mechanism by which the natriuretic hormone could increase venous tone.

5. AIM OF THE THESIS

The aim of this thesis was to develop a method for extracting and measuring endogenous Na^+K^+ATPase inhibitory activity, which could be used on a 10ml blood sample, and to use this method to investigate the hypothesis that an endogenous Na^+K^+ATPase inhibitor was present in human plasma in increased quantities in hypertensive subjects. Furthermore, the capacity of the inhibitor to cross-react with digoxin antibodies was to be measured. It was hoped that it would be possible to further investigate the factor(s) using high performance liquid chromatography.

SECTION TWO

MATERIALS AND METHODS

1. GENERAL REAGENT PREPARATION

A list of reagents used and their suppliers can be found in Appendix I. Enzymes, radio-labelled compounds and immunological reagents are included in this list. All reagents were of analytical grade.

The composition and preparation of buffers and reagent solutions are given in Appendix II.

2. PREPARATION AND STORAGE OF SPECIMENS FOR ANALYSIS

(a) Blood Samples

Blood samples from individuals were collected into lithium-heparin tubes. Plasma was separated by centrifugation (1500 x g, 20°C, 10 min) and stored at -20°C.

Samples from normotensive and hypertensive individuals were kindly provided by Dr C.G. Wathen, Department of Medicine, R.I.E.. Blood (10-20ml) was collected from newly diagnosed essential hypertensives who had never received any antihypertensive medication. Samples to be used as controls were obtained from hospital out-patients suffering from minor complaints and from volunteers; neither of these groups was on medication.

Polycythaemia is characterised by an excess of red blood cells and treatment of this condition often includes removal of relatively large quantities of blood. A large quantity of plasma was collected from a single polycythaemic patient who was also suffering from hypertension. Pooled serum collected from several patients suffering from polycythaemia was obtained from the Blood Transfusion Service.

Bovine blood, obtained in large quantities from the local abattoir, was collected into plastic containers (2l) and left at room temperature to allow it to clot. After the blood had coagulated and the clot retracted, the liquid surrounding the clot was centrifuged for 10 minutes (1500 x g, 20^{o}C) and the serum separated and stored at -20^{o}C until required. The colour of the serum indicated that there had been substantial haemolysis of the red blood cells.

(b) Bovine Adrenal Glands

Fresh bovine adrenal glands were collected onto ice and transported from the abattoir to the laboratory as quickly as possible (<30min). When the glands were to be used for the preparation of isolated cells they were transported in ice-cold normal saline (0.155M). The protocol for the preparation of cytosol from bovine adrenal cortex is given in Appendix III.

3. MEASUREMENT OF Na^+K^+ATPase ACTIVITY

In order to measure Na^+K^+ATPase inhibition, an assay was set up to measure the activity of the enzyme. Initially, an assay was used which depended on the colorimetric determination of phosphate produced by the hydrolysis of adenosine 5'-triphosphate (ATP). Subsequently, an enzyme-linked method was adapted for use on a Cobas FARA centrifugal analyser (Roche Diagnostics, Welwyn Garden City, U.K.).

(a) Colorimetric measurement of phosphate produced by ATP hydrolysis

This method comprised two parts; Na^+K^+ATPase incubation followed by the colorimetric measurement of phosphate released during the incubation.

By decreasing the enzyme concentration and increasing the incubation time proportionately, sensitivity was improved because the ratio of inhibitor to enzyme was increased and so the inhibitor would bind to a greater proportion of the total enzyme available producing more inhibition for a given inhibitor concentration. Assay conditions ranged from 6.25×10^{-3}U/l enzyme incubated for 16hr to 75U/l incubated for 1hr.

750µl imidazole buffer (pH7.8; Appendix II), 100µl ATP (200mM) and 50µl sample were pre-incubated for 10 minutes at 37°C. The incubation was started by adding 100µl Na^+K^+ATPase (dog kidney; 62.5-750U/l). After 1-16 hours 1ml of ice-cold 0.61M trichloroacetic acid (TCA) was added and 50µl portions of the mixture were taken for measurement of phosphate.

To correct for endogenous phosphate in the sample and enzyme-independent hydrolysis of ATP, controls were included with each assay batch. Three different controls were prepared; either ATP or ATPase were omitted or ATPase was added after the incubation had been terminated by the addition of 1ml TCA (0.61M). All control tubes contained buffer in place of sample.

In order to quantify the inhibition a standard curve was prepared using known concentrations of ouabain in place of sample in the incubation mixture. Ouabain standards were prepared in 10mM imidazole buffer (pH7.8; Appendix II) by diluting 2mmol/l ouabain to give 12 standards ranging from 0.005-25µmol/l.

Phosphate generated in the enzyme incubation was measured using a method based on that of Baginski and Zak (1960). This was a colorimetric method in which a phosphomolybdate complex was reduced by ascorbic acid to produce a blue

colour. Any free molybdate that remained was complexed by arsenite-citrate reagent.

Sample (50µl) was added to 1ml 0.61M TCA and incubated at room temperature for 10 minutes. After centrifugation (2800 x g, 3 min, 4°C), 500µl of the supernatant was added to 100µl 0.57M ascorbic acid. To this 250µl 8.1mM ammonium molybdate was added followed by 500µl arsenite-citrate reagent (Appendix II). After 15 minutes the absorbance was read at 700nm using a Pye-Unicam SP8-100 UV/Vis spectrophotometer (Cambridge, U.K.).

Phosphate concentrations were obtained by comparing the absorbance with a phosphate standard curve. Standards (0.07-5mM) were prepared by serial dilution of 0.5M potassium phosphate in 0.61M TCA. These covered a working range of 1-88µmol/l in the reaction mixture in the cuvette.

Precision was shown to vary depending on whether the reagents were mixed after the addition of each reagent; sequential mixing, or after all reagents had been added; terminal mixing (Fig.2.1). The slope of the line through the phosphate standards was not significantly different between the two protocols but the intercept decreased from 0.085 to 0.013 absorbance units and the precision at each point was improved. The mean coefficient of variation (CV) in sequentially mixed assays was 4.2% compared with

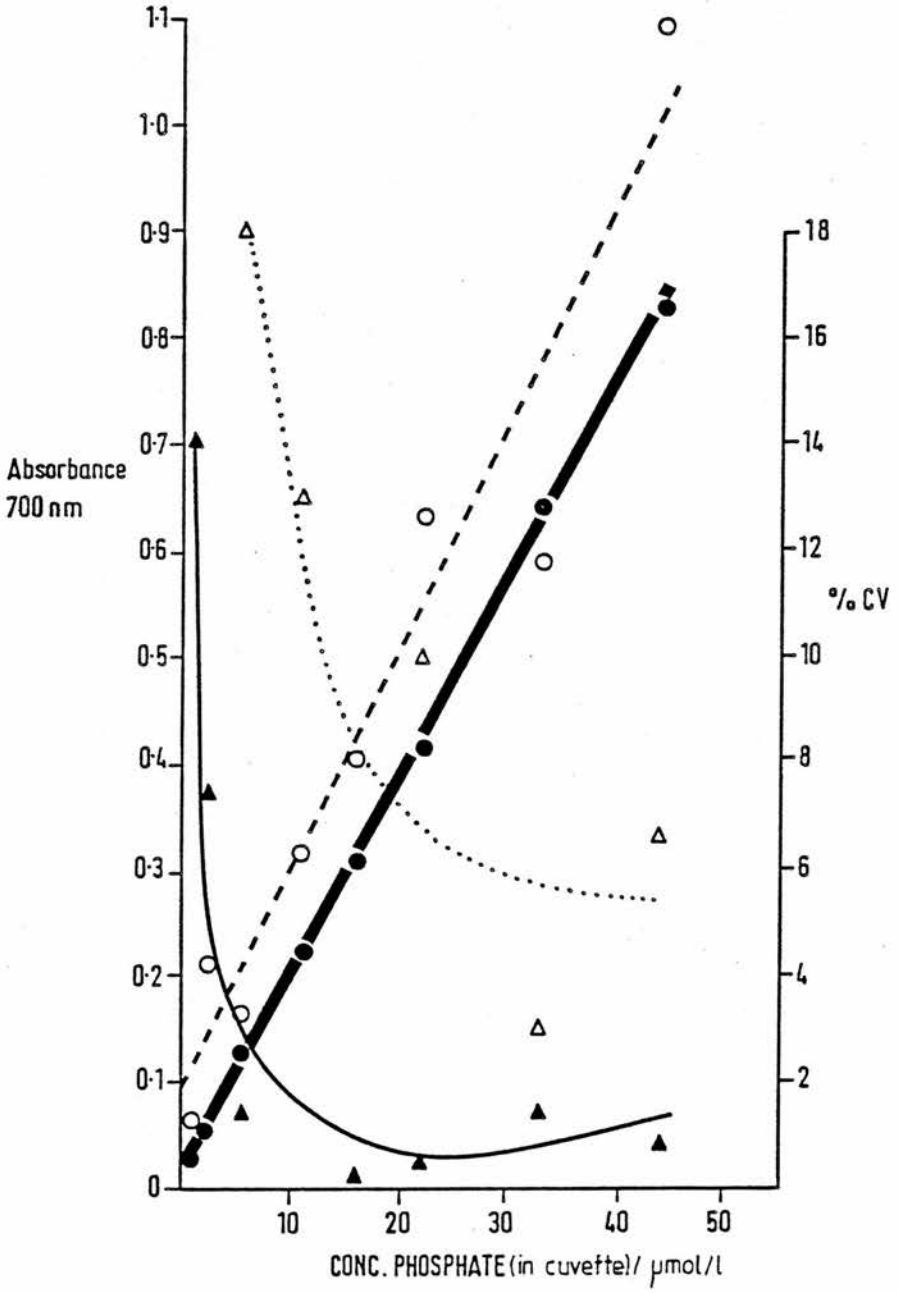

Absorbance
700 nm
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
18
16
14
12
10
8
6
4
2
% CV
10
20
30
40
50
CONC. PHOSPHATE (in cuvette) / µmol/l

FIGURE 2.1 Phosphate standard curves showing the improvement in precision when reagents were sequentially mixed.

●——● Dose response of sequentially mixed standards

o----o Dose response of terminally mixed standards

▲——▲ Precision profile of sequentially mixed standards

Δ·····Δ Precision profile of terminally mixed standards

18.2% in terminally mixed assays. The decrease in intercept of the sequentially mixed standard curve could simply be a consequence of the improved precision or may be due to the formation of a phosphate-independent complex in the terminally mixed assay.

It was observed that, if addition of arsenite-citrate reagent was delayed, a phosphate-independent complex was formed which absorbed at 700nm. There was a significant increase in the A_{700} at 2.5min in the terminally mixed assays but no significant increase in the sequentially mixed assays until 7.5min (Table 2.1). In practice the delay before addition of arsenite-citrate reagent was never more than 5min and reagents were sequentially mixed.

By improving the precision of the phosphate assay the detection limit for ouabain in the Na^+K^+ATPase incubation decreased from $5.6x10^{-6}$mol/l to $2x10^{-7}$mol/l (Fig.2.2). The detection limit is defined as the concentration of ouabain which will depress phosphate production by two standard deviations of the mean of the basal (uninhibited) value.

The interassay CV of the phosphate assay was 2.8% as determined by measurement of a 44nmol/l standard on 10 different days over a period of 6 weeks (Table 2.2). Results obtained from the assay of a 0.2µmol/l and 1µmol/l

TABLE 2.1

INCREASES IN ABSORBANCE AS A FUNCTION OF DELAYED ADDITION OF ARSENITE-CITRATE REAGENT IN SEQUENTIALLY AND TERMINALLY MIXED PHOSPHATE ASSAYS

	Absorbance at 700nm as a percentage of time zero value	
Delay time (min)	Sequentially mixed assays	Terminally mixed assays
0	100	100
2.5	99.5	133.1+
5	100.7	133.2+
7.5	103.5*	150.2+
10	102.6*	141.3+
15	107.5+	142.3+
20	113.7+	161.2+

* $p<0.05$ with respect to time zero value

+ $p<0.001$ with respect to time zero value

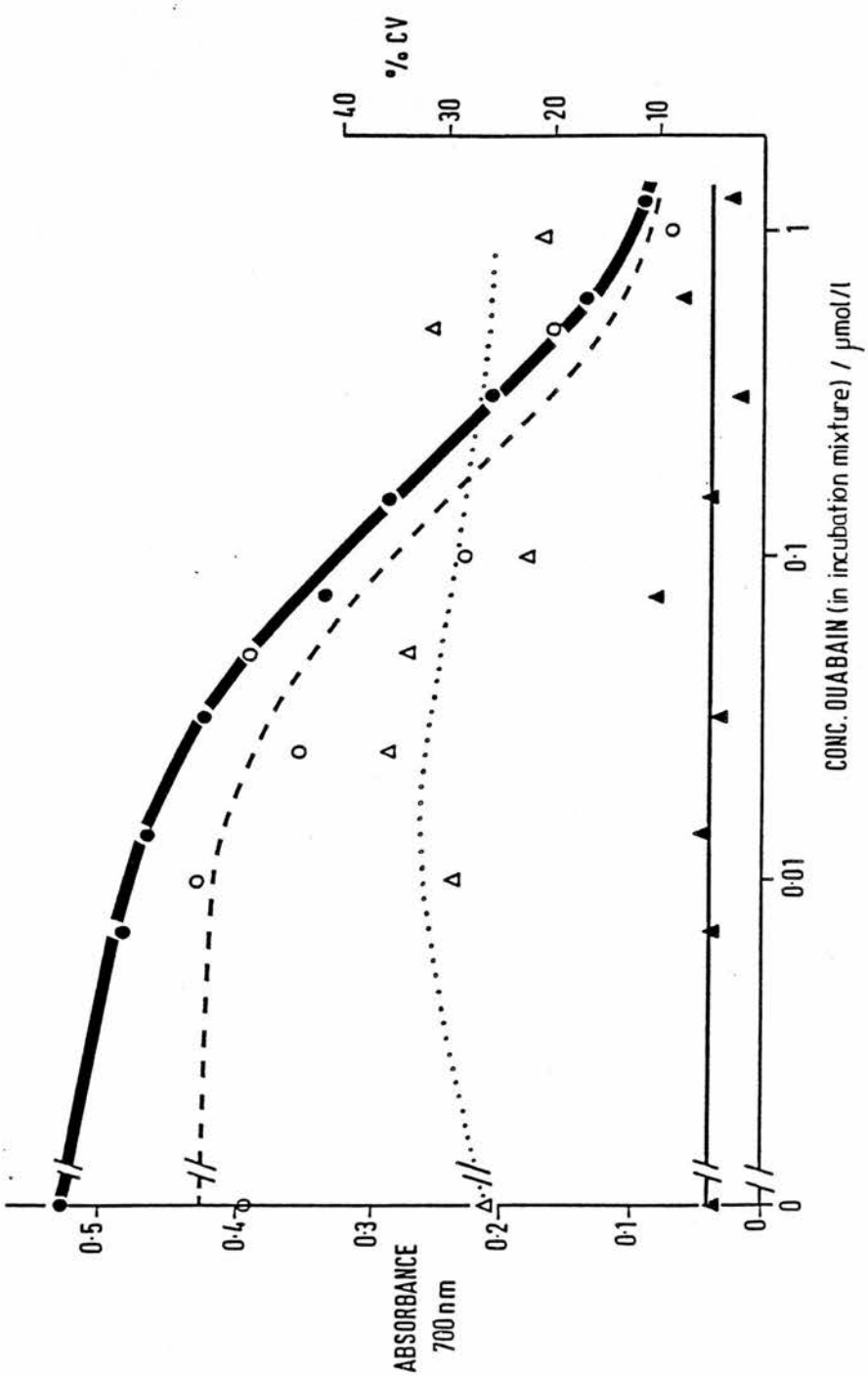

% CV
40
30
20
10
ABSORBANCE
700 nm
0·5
0·4
0·3
0·2
0·1
0
0
0·01
0·1
1
CONC. OUABAIN (in incubation mixture) / µmol/l

FIGURE 2.2 Dose-response curve for ouabain using colorimetric Na^+K^+ATPase assay showing improved precision when phosphate assay reagents were sequentially mixed. ATPase incubation conditions: 75U/l, 1h incubation, 37°C.

●———● Standard dose responses (sequential mixing)

o------o Standard dose responses (terminal mixing)

▲———▲ Precision profile (sequential mixing)

Δ·····Δ Precision profile (terminal mixing)

TABLE 2.2

INTER-ASSAY PRECISION OF THE COLORIMETRIC METHOD FOR MEASUREMENT OF PHOSPHATE

Measurement of a 44µmol/l phosphate standard on 10 different assays over a period of 6 weeks.

Mean absorbance at 700nm
0.866
0.865
0.852
0.897
0.850
0.833
0.810
0.846
0.861
0.830

MEAN = 0.851

Standard deviation = 0.024

Coefficient of Variation = 2.8%

ouabain standard in 6 consecutive assays were used to estimate the combined precision of the Na^+K^+ATPase incubation and phosphate assay (Table 2.3). A mean inter-assay CV of 8% was obtained.

(b) Enzyme-linked Na^+K^+ATPase assay of Cobas FARA centrifugal analyser.

An enzyme-linked Na^+K^+ATPase assay method (Hamlyn, et al, (1982) was adapted for use on a Cobas FARA centrifugal analyser. In this assay, hydrolysis of ATP is linked to the oxidation of NADH by pyruvate kinase and lactate dehydrogenase (Fig.2.3) and the reaction monitored by the decrease in absorbance at 340nm. The enzyme activity can be calculated by multiplying the rate of change in absorbance by a factor which is derived from the Beer-Lambert Law as shown in Figure 2.4. On the Cobas FARA the light beam passes horizontally (i.e. radially) through the cuvette so the light path is a function of the total volume of liquid present in the cuvette. It can be deduced from the calculations in Figure 2.4, that the total reaction volume can be changed without altering the calculation factor, as long as the sample volume remains the same, because the radial light path (d) cancels out of the calculation.

The complete program for the Cobas FARA is shown in Figure 2.5.

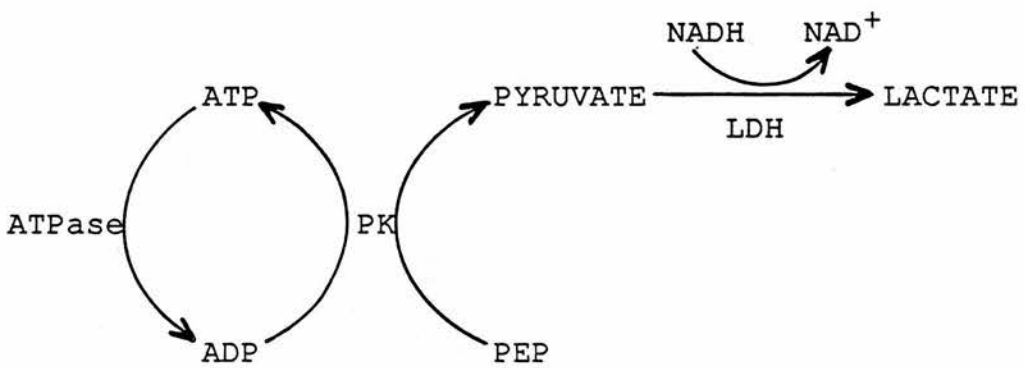
TABLE 2.3

COMBINED PRECISION OF THE Na^+K^+ATPase INCUBATION AND THE COLORIMETRIC PHOSPHATE ASSAY

Results obtained for two ouabain standards in 6 consecutive assays.

Batch Number	Inhibition of Na^+K^+ATPase (%)	
	Ouabain 0.2 µmol/l	Ouabain 1 µmol/l
1	70	45
2	63	39
3	75	48
4	80	42
5	77	46
6	68	45
MEAN	72	44
SD	6.3	3.1
CV %	9	7

FIGURE 2.3 ENZYME-LINKED Na^+K^+ATPase ASSAY SCHEME



LDH Lactate dehydrogenase

PEP Phosphoenolpyruvate

PK Pyruvate kinase

Reaction scheme of enzyme-linked assay used on Cobas FARA centrifugal analyser. Assay method based on that of Hamlyn, et al, 1982.

FIGURE 2.4 DERIVATION OF CALCULATION FACTOR FOR COBAS FARA Na^+K^+ATPase ASSAY FROM BEER-LAMBERT LAW

Cobas cuvette dimensions = r x r x d (cmxcmxcm = cm^3)
ΔA = change in absorbance at 340nm
c = concentration (mol/l)
l = light path (cm)
ε = absorption coefficient of NADH (l/mol/cm)
SV = sample volume (ml)
TV = total volume = cuvette dimensions (ml)
F = factor

$$\Delta A = \varepsilon c l$$

$$\Delta A \times \frac{1}{\varepsilon x l} = c \qquad \text{(mol/l)}$$

l = d cm

$$\Delta A/min \times \frac{1}{\varepsilon x d} = c/min \qquad \text{(mol/l/min)}$$

$$\Delta A/min \times \frac{1}{10^{-6}} \times \frac{1}{\varepsilon x d} = c/min \qquad (\mu mol/l/min = U/l)$$

Dilute sample: SV/TV ∴ multiply by TV/SV

$$\Delta A/min \times \frac{10^6}{\varepsilon x d} \times \frac{TV}{SV} = c/min \qquad (U/l)$$

$$\Delta A/min \times \frac{10^6}{\varepsilon x d} \times \frac{r^2 d}{SV} = c/min \qquad (U/l)$$

$$\Delta A/min \times \frac{10^6}{\varepsilon} \times \frac{r^2}{SV} = c/min \qquad (U/l)$$

$\varepsilon = 6.3x10^3$ l/mol/cm;
r = 0.5cm;
SV = 20µl = 0.020ml = $0.020cm^3$;

So:

$$\Delta A/min \times \frac{10^6}{6.3x10^3} \times \frac{(0.5)^2}{0.02} = \Delta A/min \times F \qquad (U/l)$$

∴ F = 1984

FIGURE 2.5

ENZYME-LINKED Na^+K^+ATPase ASSAY PROGRAM FOR COBAS FARA CENTRIFUGAL ANALYSER

GENERAL

```
MEASUREMENT MODE     :  ABSORB
REACTION MODE        :  P-I0-SR1-AO

CALIBRATION MODE     :  FACTOR
REAGENT BLANK        :  NO BLANK

WAVELENGTH           :  340 nm
TEMPERATURE          :  37.0°C

DECIMAL POSITION     :  1
UNIT                 :  U/l
```

ANALYSIS

```
P     SAMPLE     :   20 µl  DILUENT:   40 µl
      REAGENT    :  150 µl
I0    INCUBATION:   20 s
SR1   START R 1 :    20µl  DILUENT:   20 µl
A0    READINGS
        FIRST    :  0.5 s  NUMBER :   40
        INTERVAL:    5 s
```

CALCULATION

```
CONVERS.FACTOR : 1.00000 OFFS.:0.00000
REAC.DIRECTION : DECREAS     CHECK: ON
SAMPLE LIMIT   : NO

TEST RANGE   LO: NO    HI  NO      U/l
NORM.RANGE   LO: NO    HI  NO      U/l

CALC. STEPS    : 1
CALC. STEP A   : KINSEARCH
READINGS FIRST : 12          LAST: 40
REACT. LIMIT   : NO
```

CALIBRATION

```
FACTOR         : 1984.00
```

Briefly, 20µl of sample or ouabain standard, plus 40µl of diluent (H_2O), and 150µl FARA assay reagent (Appendix II) were pipetted, mixed by centrifugation and pre-incubated for 20s at 37°C. 20µl of Na^+K^+ATPase (dog kidney, Sigma; 500U/l) and 20µl diluent were then pipetted, mixed and incubated for 200s at 37°C. During the incubation 40 absorbance readings were taken, one every 5 seconds after an initial reading at 0.5 seconds.

Enzyme activity was calculated over the period 60-200s using the "kinsearch" calculation mode. In this mode, the program will "search" for the linear part of the reaction by drawing lines to each data point from a point equal in absorbance to but 5s prior to the first absorbance measurement. Beginning with the last data point, the tangent of the angle formed between the line and the horizontal is calculated. When the tangent is less than or equal to the previous tangent the data point from which the line has been drawn is taken as the end of the linear range. The beginning of the linear range is defined in the same way, by drawing lines from a point 5s prior to the last data point but equal in absorbance and calculating the tangent of the angle with the horizontal. The change in absorbance per minute is calculated by linear regression analysis of the points between the beginning and the end of the linear range. If there are fewer than four data points within the linear range, the program will not

perform the calculation procedure on the data points and will "flag" the sample "NON-LINEAR". The program also checks the scatter of the data points within the linear range. If one or more points lies outside the allowed limit of +/-0.005 absorbance units the progam performs the calculation on the data points but prints "NOISE" next to the value on the results.

Inhibition caused by a sample was converted to ouabain equivalents by comparison with a graph of percentage inhibition of Na^+K^+ATPase versus ouabain concentration, i.e. a ouabain standard dose-response curve. The concentration range of the ouabain standards was 0.25-1000μmol/l.

As shown in Figure 2.6, the absorbance change in the presence of different standards has a wide range. A compromise had to be reached where the zero standard i.e. no inhibitor present, did not use all the NADH but where the ouabain standards in the middle of the curve gave a linear result. The time window chosen for calculating activity was 60-200s.

The assay performed well with an intra-assay CV of no greater than 2% and an inter-assay CV with a mean of 4.5%. The inter-assay CV over 6 consecutive assays shows a transient rise at 250μmol/l (Fig.2.7) which is due to the

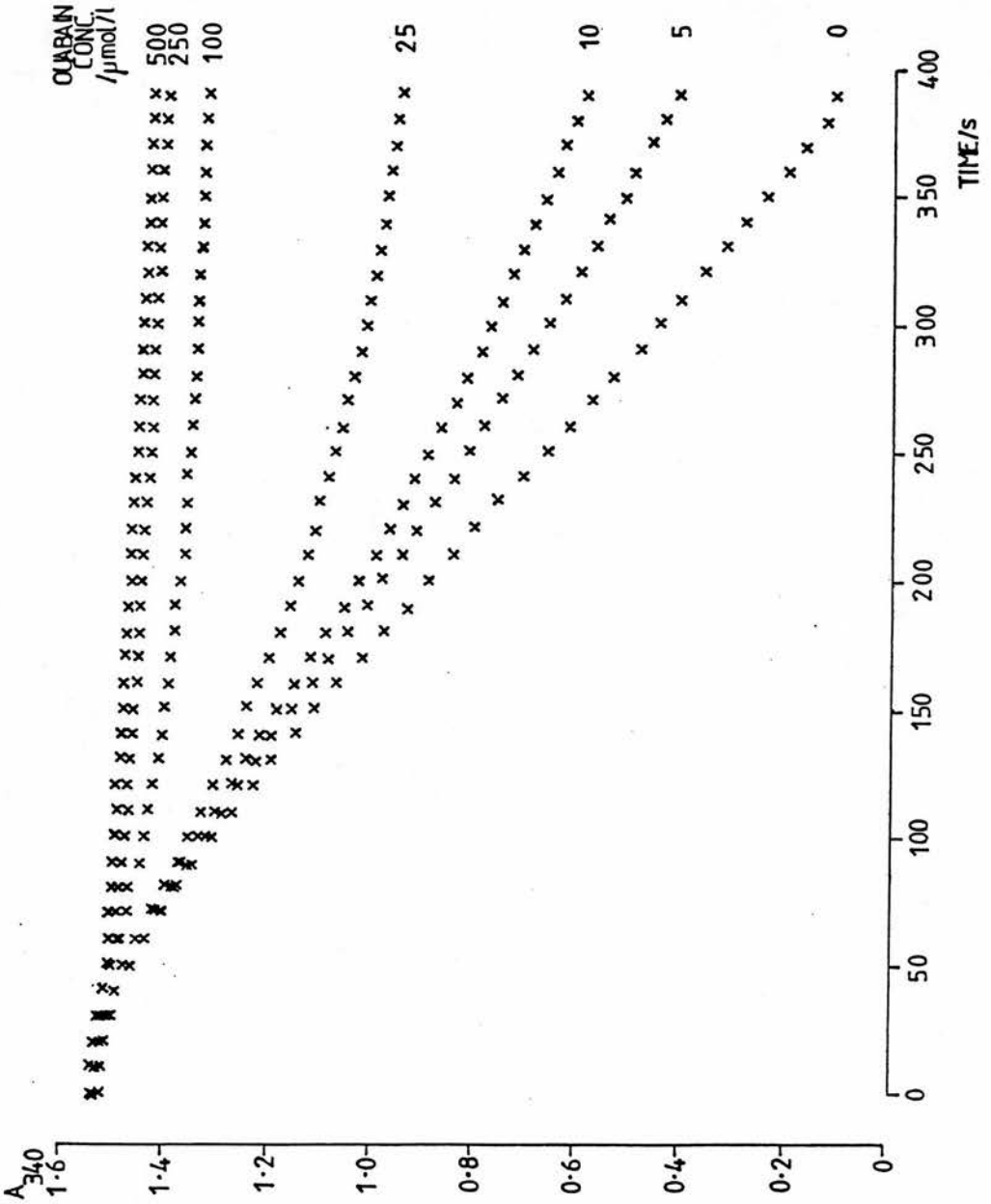

OUABAIN CONC /μmol/l
500
250
100
25
10
5
0
A_{340}
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
50
100
150
200
250
300
350
400
TIME/s

FIGURE 2.6 Enzyme-linked Na^+K^+ATPase assay on Cobas FARA. Change in absorbance at 340nm in the presence of different ouabain standards.

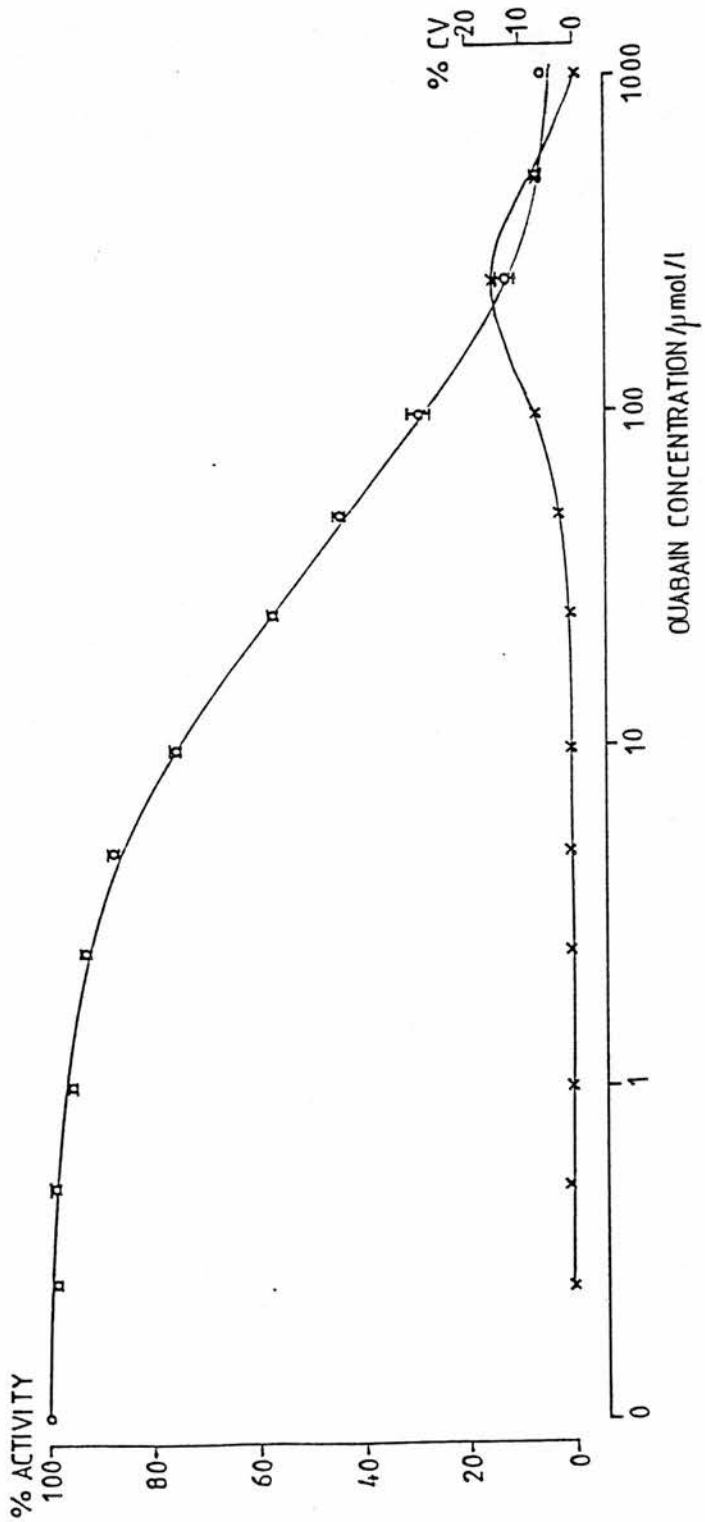

% ACTIVITY
100
80
60
40
20
0
% CV
20
10
0
0
1
10
100
1000
OUABAIN CONCENTRATION /µmol/l

FIGURE 2.7 Typical dose-response curve for ouabain using the enzyme-linked Na^+K^+ATPase assay.

o——o Standard dose responses

×——× Precision profile - interassay CV over six consecutive assays.

decrease in the slope of the dose-response curve at that point without a concomitant fall in the variation of the response. At ouabain concentrations greater than 250μmol/l the variation in response was much less because the concentration of ouabain was approaching that which causes maximal inhibition of the enzyme and the curve approached an asymptote.

4. MEASUREMENT OF DIGOXIN-LIKE ACTIVITY

Endogenous cardiac glycoside-like substances were expected to be sufficiently similar to digoxin in structure that they would be capable of cross-reacting with digoxin antibodies. Eluates were investigated for cross-reactivity with several digoxin radioimmunoassays; the department of Clinical Chemistry "in-house" assay which is used for routine measurement of patient samples and two commercial kits.

(a) Commercial Digoxin Radioimmunoassay Kits

The kits were used as directed by the manufacturer's instructions. Each kit used a different method to separate the antibody-bound [^{125}I]-digoxin from the free [^{125}I]-digoxin.

(i) Gammacoat^{TM}[^{125}I]-Digoxin Radioimmunoassay Kit

(Clinical Assays, Massachusetts, U.S.A.)

The tubes used in this assay were pre-coated with anti-digoxin antibody so separation of bound from free [^{125}I]-digoxin was achieved by simply decanting the liquid, which contained the free digoxin, from the tubes.

[^{125}I]-digoxin was diluted with phosphate buffered saline (supplied with the kit), to give a 1.48kBq/100ml solution. 50µl of sample and 1ml of [^{125}I]-digoxin solution were pipetted into each tube. After a one hour incubation at 37^{o}C the liquid was decanted and the tubes counted on a gamma counter.

(ii) RIANEN^{TM}[^{125}I]-Digoxin Radioimmunoassay Kit

(New England Nuclear, Massachusetts, U.S.A.)

A prereacted first and second antibody complex is used to separate antibody-bound [^{125}I]-digoxin from free [^{125}I]-digoxin.

[^{125}I]-digoxin was supplied as a dilute solution in acetate buffer (1.6kBq/ml). 100µl sample, 500µl [^{125}I]-digoxin solution and 500µl antibody complex were incubated at room temperature. After 30 minutes, the tubes were centrifuged (1000 x g; 10 min) and the supernatant decanted by gently inverting the tubes.

(b) "In-house" method

The immunological reagents required for this assay were supplied by the Scottish Antibody Production Unit (SAPU). [^{125}I]-digoxin was obtained initially from CMD (UK) Ltd (Bournemouth, U.K.) and latterly from Ciba Corning Diagnostics Ltd (Halstead, Essex, U.K.). This was diluted with phosphate buffer (pH 7.2; appendix II) to a concentration of approximately 1kBq/ml. Separation of bound [^{125}I]-digoxin was achieved using a pre-precipitated antibody suspension (Appendix II) which must be stirred continuously during dispensing. Samples and reagents were pipetted with a Dilutrend diluter (Clinicon Mannheim GMBH). 50µl sample, 400µl [^{125}I]-digoxin solution and 400µl pre-precipitated antibody complex were incubated for 30 minutes. The tubes were then centrifuged (1000 x g; 20 min; 4^{o}C) and the supernatant decanted leaving a pellet of antibody-bound [^{125}I]-digoxin.

(c) Effect of sample diluent on "in-house" method

The results from the digoxin RIA were shown to be affected by the sample matrix, a widely observed occurrence in radioimmunoassays for steroids. Samples were to be reconstituted in ammonium acetate but digoxin standards diluted in ammonium acetate produced a curve which was parallel to the serum standard curve but displaced to the

right (Fig.2.8). Bovine serum albumin (BSA) was added to each assay to increase the protein concentration to the level obtained when a serum sample is added. This produced a curve which was parallel to the buffer curve but did not return the curve to the same position as the serum curve (Fig.2.9).

Although digoxin standards diluted in ammonium acetate produced curves which had acceptable within batch variation the between batch variation was less acceptable (Fig.2.8) and the ammonium acetate control which was read from the curves had a CV of 67% (Table 2.4).

The buffer used in the enzyme-linked Na^+K^+ATPase assay; Tris-TES-salts (TTS), was also a possible candidate for reconstitution of samples, digoxin standards were prepared using TTS as the diluent. As with ammonium acetate, the precision within a batch was adequate but between batches the CV was greater than 10% for digoxin concentrations above 1nmol/l (Fig.2.8). Controls prepared in TTS had CV's greater than 10% (Fig.2.9).

When standards were diluted in digoxin RIA buffer a curve was obtained which was very similar to the serum curve. Precision was as good as the serum curve (Fig.2.8) and the controls had acceptable CV's (Table 2.4). Unlike the other buffer standard curves, the RIA buffer standard curve

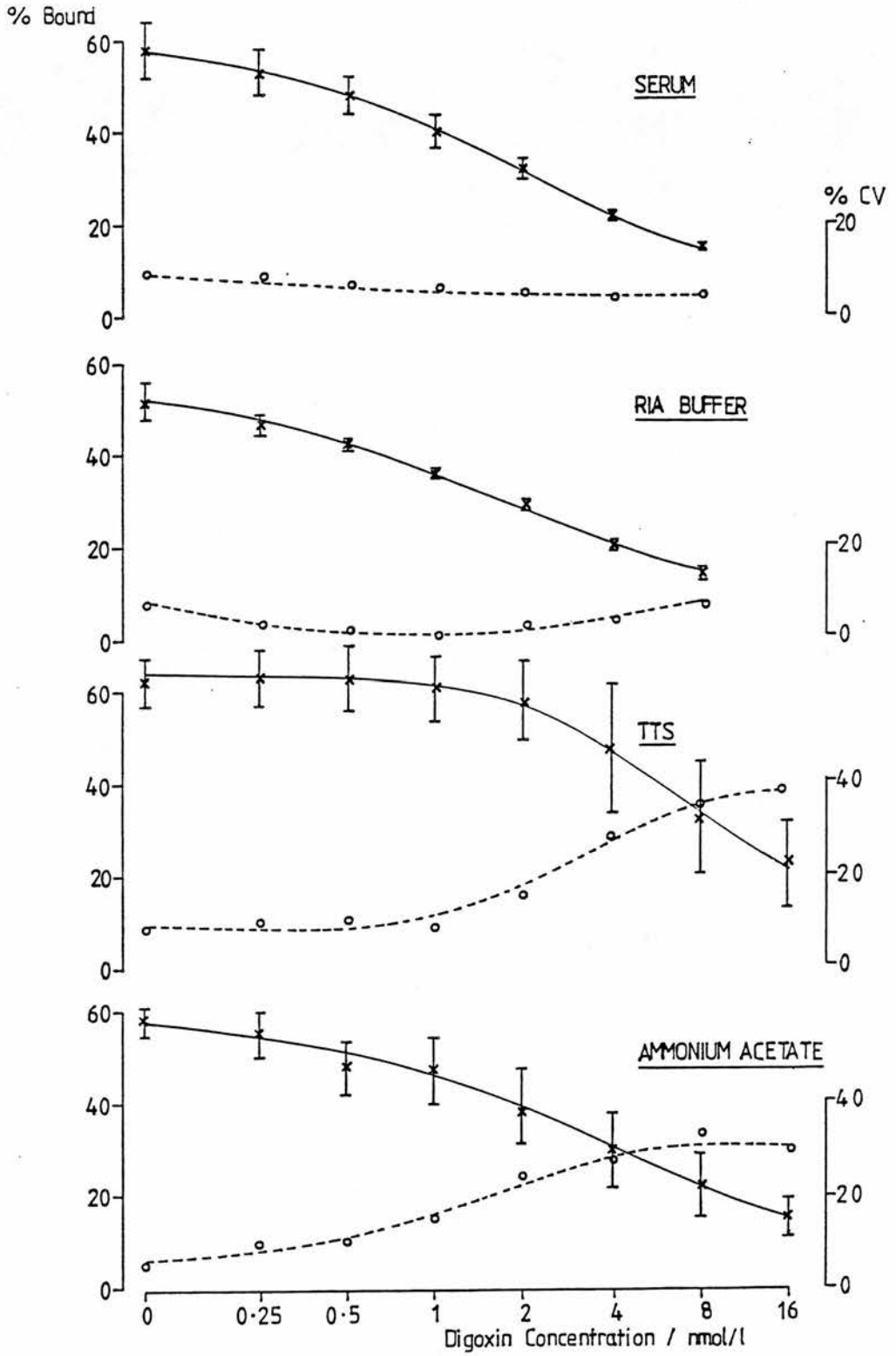
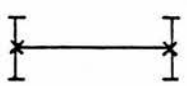

% Bound
% CV
SERUM
RIA BUFFER
TTS
AMMONIUM ACETATE
0
20
40
60
0·25
0·5
1
2
4
8
16
Digoxin Concentration / nmol/l

FIGURE 2.8 Dose-response curves for digoxin RIA using different diluents.



Mean and standard deviation of response at each dose over six consecutive assays

o ------o Interassay precision profile

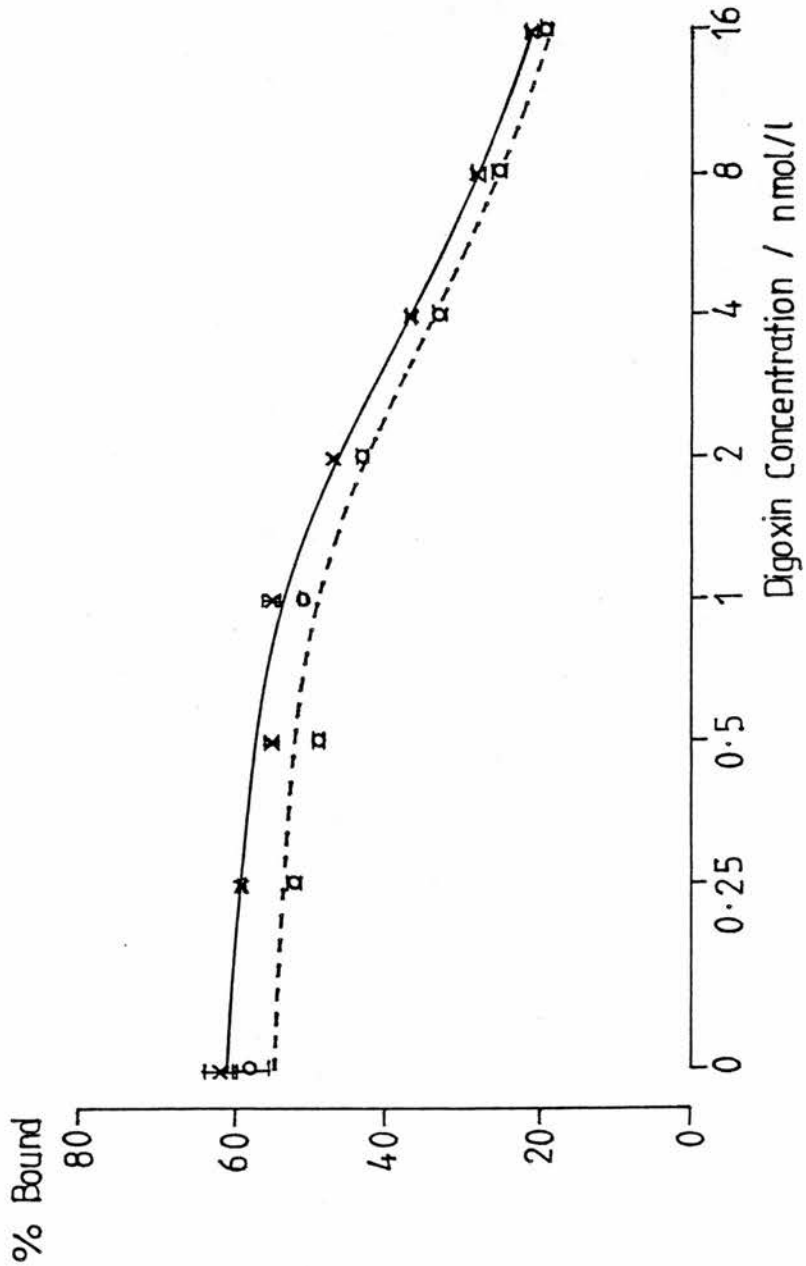
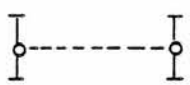

% Bound
80
60
40
20
0
0
0·25
0·5
1
2
4
8
16
Digoxin Concentration / nmol/l

FIGURE 2.9 Effect of addition of BSA to assay tubes containing standards diluted in ammonium acetate.

Mean and standard deviation of response (no additional BSA)



Mean and standard deviation of response (+ BSA)

TABLE 2.4

CONTROL VALUES FOR DIGOXIN RADIOIMMUNOASSAY

Diluent	No. of Assays	Digoxin Concentration nmol/l		
		Mean	SD	%CV
Ammonium acetate	10	5.8	3.9	67
TTS	10	2.4	0.48	20
		3.5	0.45	13
		6.3	0.82	13
RIA Buffer	10	0.6	0.09	15
		3.7	0.31	8
		6.8	0.43	6
Serum	10	2.4	0.13	5
		5.1	0.5	10

was not displaced relative to the serum curve. Therefore, digoxin standards were prepared using RIA buffer as the diluent.

5. EXTRACTION AND SEPARATION METHODS

(a) Extraction using Sep-Pak C_{18} Cartridges

(i) Method

Waters Sep-Pak octadecylsilane (C_{18}) cartridges are intended for sample preparation prior to further liquid chromatography. The cartridges are first primed with a solvent less polar than water, then washed with water prior to addition of the sample. This is followed by a water wash to flush out inorganic ions and then by a solvent or series of solvents which become increasingly non-polar. Compounds elute in order of decreasing polarity.

Sep-Pak cartridges were used to concentrate the methanol-soluble fraction from human plasma, human serum, bovine serum and bovine adrenal cortex cytosol. A similar protocol was used for each sample. Liquids were pushed through the cartridges using either nylon syringes under hand pressure or using a Sep-Pak cartridge rack (Millipore U.K., Harrow, Middlesex, U.K.) linked to a vacuum pump. Each cartridge was primed with 5ml of methanol followed by 5ml of distilled water before the sample was applied. The

fraction of the sample which did not bind to the octadecylsilane was described as non-adsorbed filtrate (NAF). After sample application the cartridge was rinsed with 5-10ml of distilled water followed by 3-5ml of methanol. Substances which eluted with methanol were called eluate. Eluates were either collected into Pyrex conical tubes (13ml) or collected into 5ml glass tubes and transferred to polypropylene tubes (5ml) depending on which drying apparatus was to be used.

Initially, eluates were dried down using a water bath to heat the tubes and a manifold of plastic tubes joined by rubber and plastic tubing to a compressor or a nitrogen cylinder to blow air into them. More recently, a Techne sample concentrator (Techne (Cambridge) Ltd., Cambridge, U.K.) was purchased which performed the same task in a more sophisticated manner (Fig.2.10). The eluates were dried down at 37°C or 50°C.

When it was necessary to achieve a greater degree of concentration than could be obtained by a single extraction, the eluate was extracted on a second cartridge. The eluate from the repeat extraction was termed concentrated eluate.

(ii) Recovery Experiments

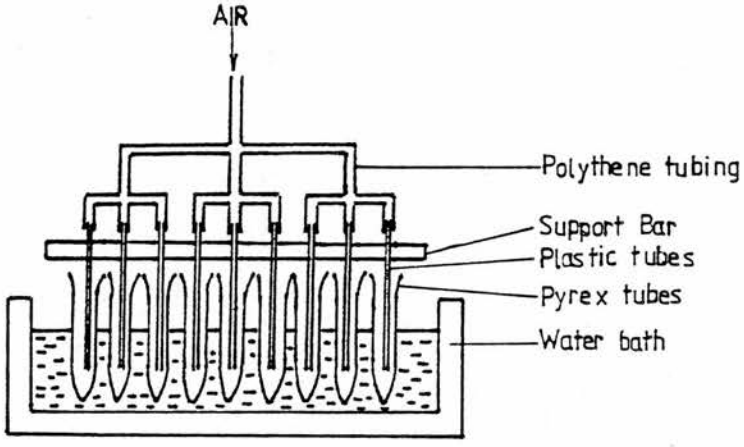
Ouabain was used as a model in experiments to investigate

FIGURE 2.10

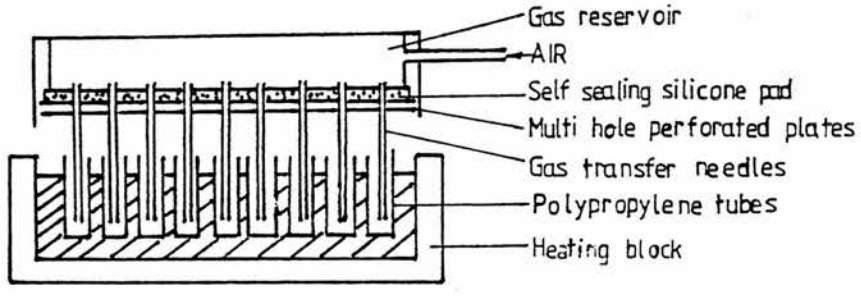
APPARATUS USED TO DRY DOWN SEP-PAK ELUATES

Air was supplied from a compressor.

"In-house" Apparatus



Techne sample concentrator



the recovery of inhibitory substances using this extraction method. When the enzyme-linked Na^+K^+ATPase assay was introduced the performance of the method was re-evaluated.

Initially recovery was assessed using the colorimetric Na^+K^+ATPase assay. The methanolic eluate was collected into conical 13ml Pyrex tubes and dried down under air, in a water bath at 50°C. The main problem with this method was the small number of positions available on the drying apparatus which limited the number of samples which could be extracted, dried and assayed on any one day.

The effect of the methanol elution volume on recovery was investigated by loading cartridges with 5ml of 1µmol/l ouabain in NAF. The NAF was used because it was a serum-like matrix which should not contain any endogenous Na^+K^+ATPase inhibitor. The cartridges were eluted with 1-10ml of methanol. The recovery varied between 70 and 120% for elutions volumes up to 5ml but was less than 70% for volumes greater than 5ml (Fig.2.11). Recoveries greater than 100% were due to variation in the assay resulting in the measurement of slightly more ouabain than was added. The lower recoveries for elution volumes of 6ml and 10ml may be due to losses on the side of the tubes. An elution volume of 5ml was chosen to give the maximum possible chance of extracting all bound material.

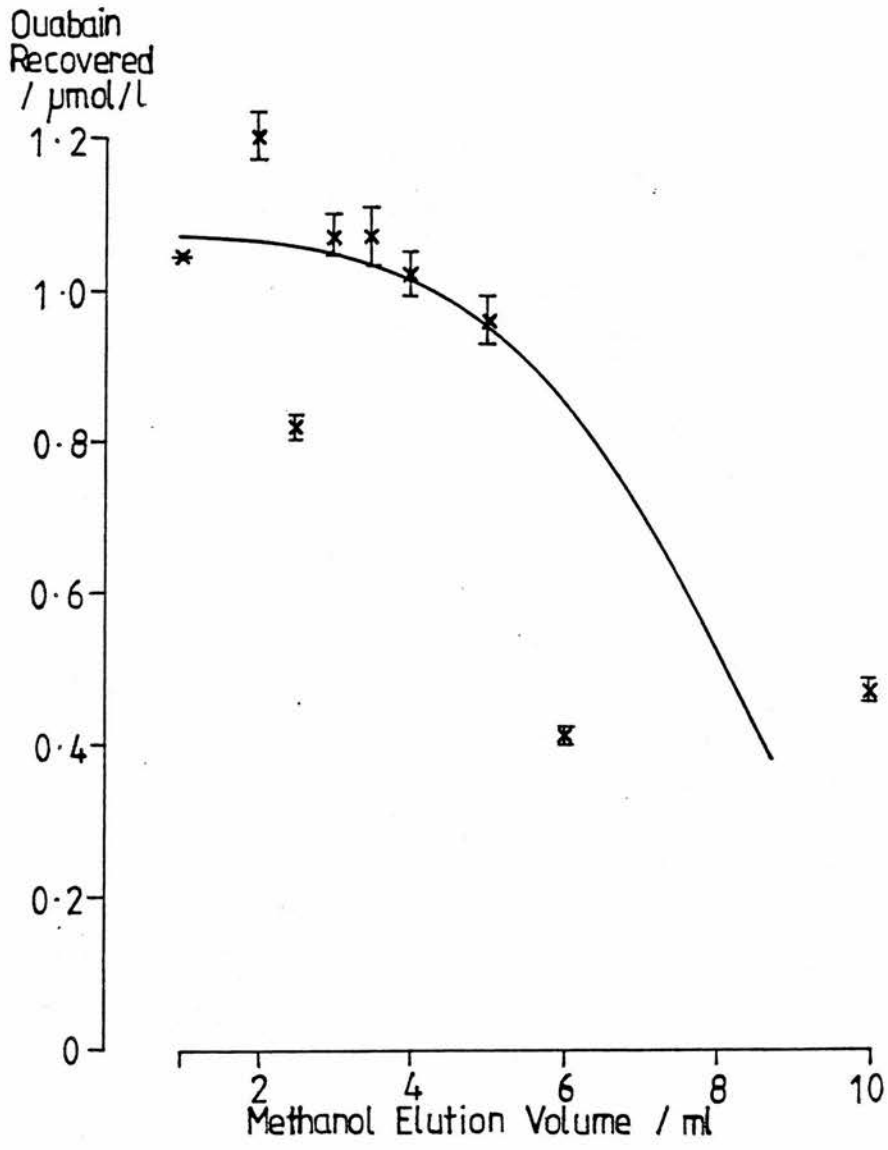


FIGURE 2.11 Sep-Pak extraction method - effect of methanol elution volume on recovery of ouabain. Ouabain added = 1µmol/l.

Mean and standard deviation of triplicate incubation (colorimetric Na^+K^+ATPase assay)

Recovery did not appear to be affected by sample volume. In an experiment where the loading volume was 2.5, 5, or 10ml the recovery was not related to loading volume (Table 2.5). This experiment also confirmed that ouabain was only detected in the eluate and not in the NAF or in the water wash.

The use of a Techne sample concentrator and the development of the enzyme-linked Na^+K^+ATPase assay on the Cobas FARA made it necessary to repeat the recovery experiments because the eluate was dried down in different tubes and a different Na^+K^+ATPase assay was being used.

Recovery experiments were performed using ouabain diluted in NAF as a model. In all cases the cartridges were primed with 5ml methanol followed by 5ml water and rinsed with 5ml water prior to elution with methanol.

The maximum volume of the tubes for the drying apparatus was 5ml. The effect of varying the methanol elution volume was investigated and it was observed that recovery was not compromised if 3ml of methanol was used (Fig.2.12).

TABLE 2.5

SEP-PAK EXTRACTION METHOD: EFFECT OF SAMPLE VOLUME ON RECOVERY

Sample Volume (ml)	Recovery (%)	Mean	SD
10	110 115	112	3.5
5	89 85	87	2.8
2.5	156 140	148	11.3

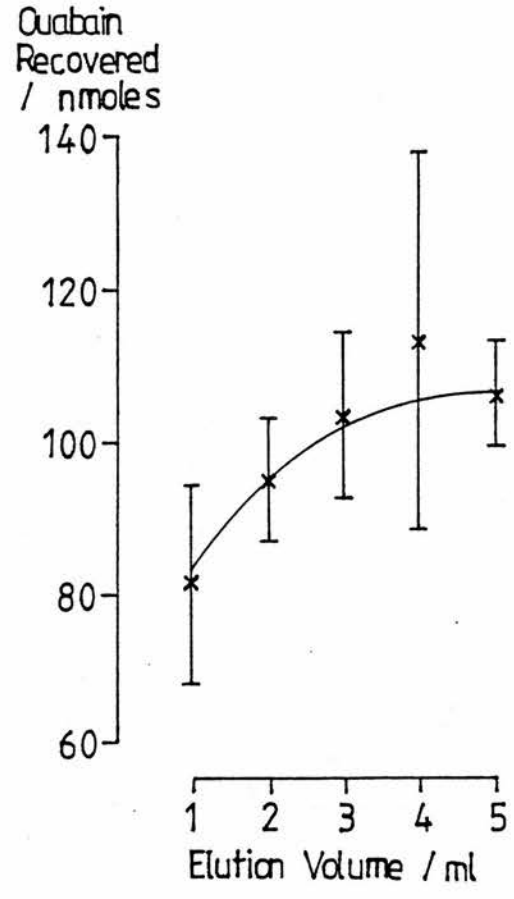
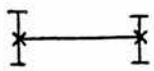


FIGURE 2.12 Sep-Pak extraction method - effect of methanol elution volume on recovery of ouabain. Ouabain added = 100nmoles.



Mean and standard deviation of duplicate extraction, single estimation of ouabain concentration (enzyme-linked Na^+K^+ATPase assay)

Recovery was not affected by varying the sample volume (Fig.2.13) although the values were greater than 100% throughout the range. The recoveries greater than 100% may have been due to the use of NAF as a diluent. It is possible that not all of the inhibitory factor had been removed from the NAF and so the inhibition measured would have been due to the ouabain which had been added and any remaining factor present in the NAF.

Comparable recoveries were observed for reconstitution volumes of 200µl to 1ml (Fig.2.14). A reconstitution volume of 100µl had a recovery which was greater than 100% which may have been due to the presence of an inhibitory factor in the NAF, as discussed above.

(b) Separation using reverse-phase high performance liquid chromatography

Concentrated eluates were separated into several active fractions using reverse-phase high performance liquid chromatography (HPLC). The HPLC system was obtained from Waters (Instruments) Ltd. (Northwich, Cheshire) and comprised a Waters Model 680 automated gradient controller, two Waters Model 510 pumps, a Model 441 absorbance detector and a Model U6K sample injector. The detector and controller were connected to a BBC SE120 pen recorder (BBC Metrawatt/Goerz, Colorado, U.S.A.). HPLC grade solvents

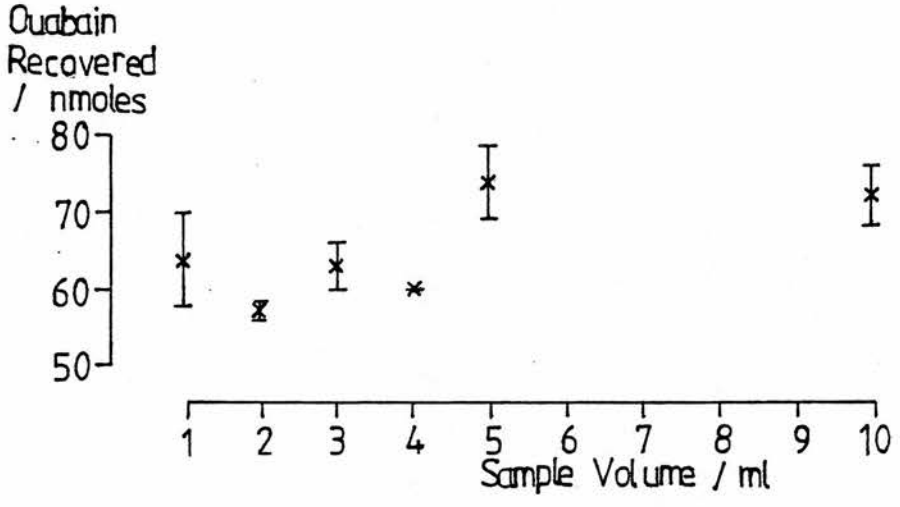


FIGURE 2.13 Sep-Pak extraction method - effect of sample volume on recovery of ouabain. Ouabain added = 50nmoles.

Mean and standard deviation of duplicate extraction, single estimate of ouabain concentration (enzyme-linked Na^+K^+ATPase assay)

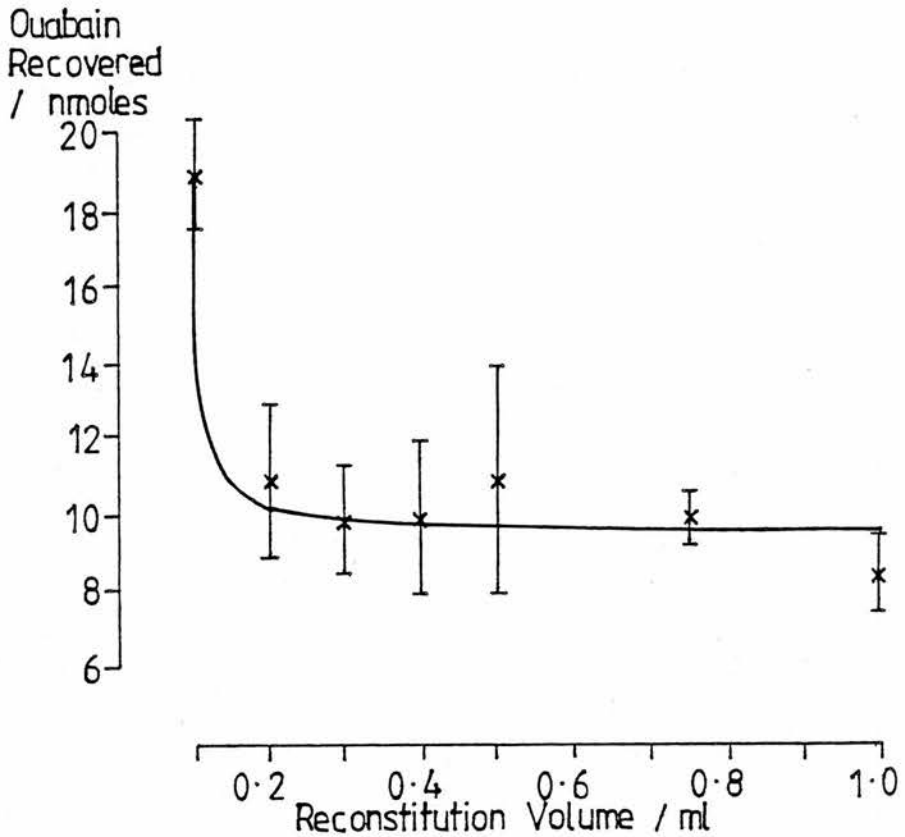
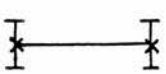


FIGURE 2.14 Sep-Pak extraction method - effect of reconstitution volume on recovery of ouabain. Ouabain added = 10nmoles.



Mean and standard deviation of duplicate extraction, single estimate of ouabain concentration (enzyme-linked Na^+K^+ATPase assay)

were filtered before use using Waters filtration apparatus and 0.5µm filters.

After samples had been microfuged to remove particulate matter, they were applied to a Waters µBondapak octadecylsilane column (3.9mm x 30cm; Waters Associates, Harrow, Middlesex) and eluted with a 20-100% acetonitrile gradient at a flow rate of 1ml/min. 1ml fractions were collected with a LKB Superrac 2211 fraction collector (LKB, Bromma, Sweden) and dried down under air at 37°C using the Techne sample concentrator. Fractions were reconstituted in 250µl 20mM ammonium acetate (pH 7.4; Appendix II) and assayed for the ability to inhibit Na^+K^+ATPase and to cross-react with digoxin antibodies.

6. PREPARATION OF ISOLATED ADRENAL CELLS AND THEIR INCUBATION WITH AGONISTS

(a) Preparation of isolated cells

Adrenal cells were prepared from bovine adrenal glands using a method similar to that of Crivello, et al, (1982).

Bovine adrenal glands were collected from the abattoir and transported to the laboratory in ice-cold 0.155M saline where the fat was initially quickly trimmed and then carefully dissected from the glands. The glands were kept

moist, with saline or Earle's balanced salt solution (EBS; NBL Ltd., Cramlington, Northumberland, U.K.), throughout this procedure. 0.2%BSA and 0.1%glucose were added to EBS and medium 199 (M199; Flow Laboratories, Rickmansworth, Herts., U.K.) which brought the total glucose concentration to 0.2%. Slices were taken from the outside of the glands using a Staddie-Rigg microtome; the first slice was considered to contain predominantly glomerulosa cells, the second slice predominantly fasciculata cells. Cells were prepared in parallel from these two types of slice. The slices were chopped into extremely small pieces and incubated in 2%BSA-EBS containing 1.5mg/ml collagenase for 1hr. The mixture was dispersed every 20min using a 5ml "finnpipette" with a tip with a cut end. Digested material was passed through 100μm nylon gauze, centrifuged (100 x g, 15min, 20°C), and the pellet resuspended in medium 199. This washing procedure was repeated a further three times. After the centrifuging for the fourth time the resuspended pellet was passed through a 20μm nylon gauze and the cell suspension diluted to the required volume.

Cells were counted using a cytometer. The number of cells in a 4x4 grid multiplied by $1x10^4$ was equivalent to the number of cells per ml of suspension. Cells were counted routinely and the average value of the number of cells present in each of the four 4x4 grids was calculated.

(b) Incubation of isolated adrenal cells with agonists

The isolated adrenal cells were tested for their steroidogenic response to AII, ACTH, and to the active fractions from the HPLC separation of adrenal cortex cytosol eluate.

Cell suspension (225µl) was incubated for 1hr at 37°C in the presence of 25µl agonist and 250µl M199 in a shaking waterbath. The incubate was centrifuged (200 x g, 15min, 4°C) and the supernatant decanted into fresh tubes (3ml) and stored at -20°C prior to measurement of steroid concentration by radioimmunoassay.

7. RADIOIMMUNOASSAY OF ADRENAL STEROIDS

Like the cells of the human adrenal gland, bovine glomerulosa cells produce predominantly aldosterone and fasciculata cells produce predominantly cortisol; both types of cell produce corticosterone. In order to assess whether the active fractions from HPLC had a differential effect on the steroid production in both types of cell the concentration of aldosterone, corticosterone and cortisol in the supernatants from the cell incubations was measured using appropriate radioimmunoassays. Assays were pipetted using the same automatic diluter used for the digoxin radioimmunoassay and all standards were diluted in 0.2%BSA-EBS.

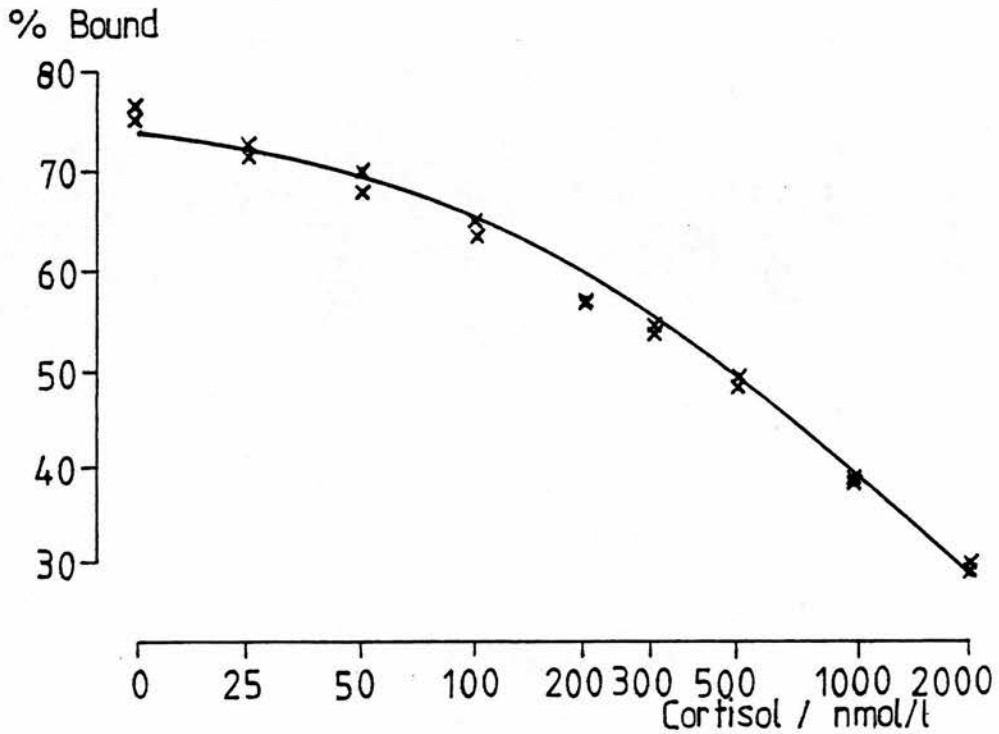
(a) Cortisol Radioimmunoassay

Cortisol concentrations were measured using an "in-house" cortisol radioimmunoassay which uses immunological reagents provided by SAPU. In this method, [^{125}I]-cortisol (Amersham International plc, Amersham, Bucks., U.K.) was diluted with 0.1M citrate buffer (pH 4; Appendix II) to a concentration of 330Bq/ml.

25µl of sample and 400µl of [^{125}I]-cortisol followed by 250µl of pre-precipitated cortisol antibody (Appendix II) were pipetted into assay tubes and incubated at 37^{o}C. After 70 minutes the tubes were centrifuged (3000 x g; 30min) and the supernatant decanted. Cortisol standards were prepared from 10mM cortisol stock, in ethanol, by dilution in EBS containing 0.2%BSA and stored at -20^{o}C. The standards covered the range 25-2000nmol/l.

The cortisol concentrations in some of the cell preparations were quite low so the assay was sensitised by increasing the sample volume to 100µl and diluting the pre-precipitated antibody two fold. An alternative set of standards were prepared for use with this more sensitive assay. These standards covered the range 1-2000nmol/l.

Typical dose-response curves for the two assays are shown in Figures 2.15 and 2.16.


% Bound
80
70
60
50
40
30
0
25
50
100
200
300
500
1000
2000
Cortisol / nmol/l

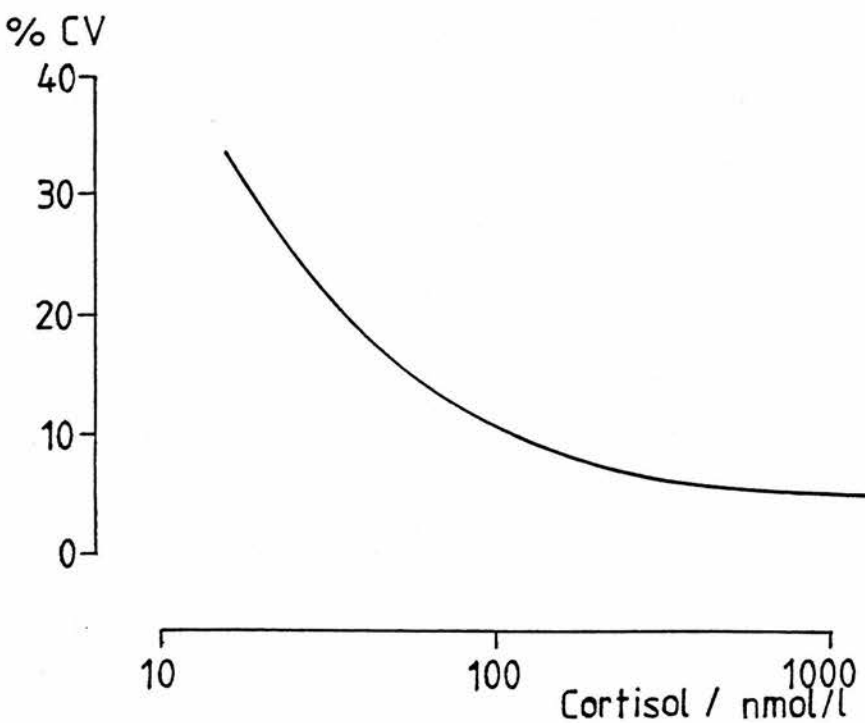
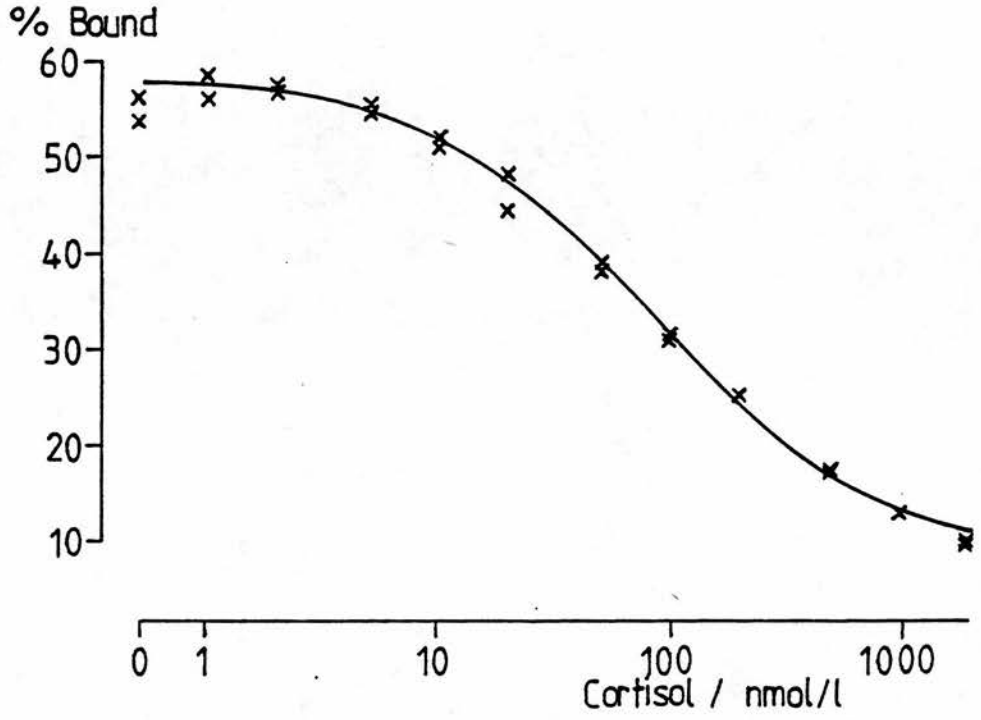

% CV
40
30
20
10
0
10
100
1000
Cortisol / nmol/l

FIGURE 2.15 Typical dose-reponse curve and precision profile for the "in-house" method cortisol RIA, as calculated by the WHO immunoassay program.


% Bound
60
50
40
30
20
10
0
1
10
100
1000
Cortisol / nmol/l

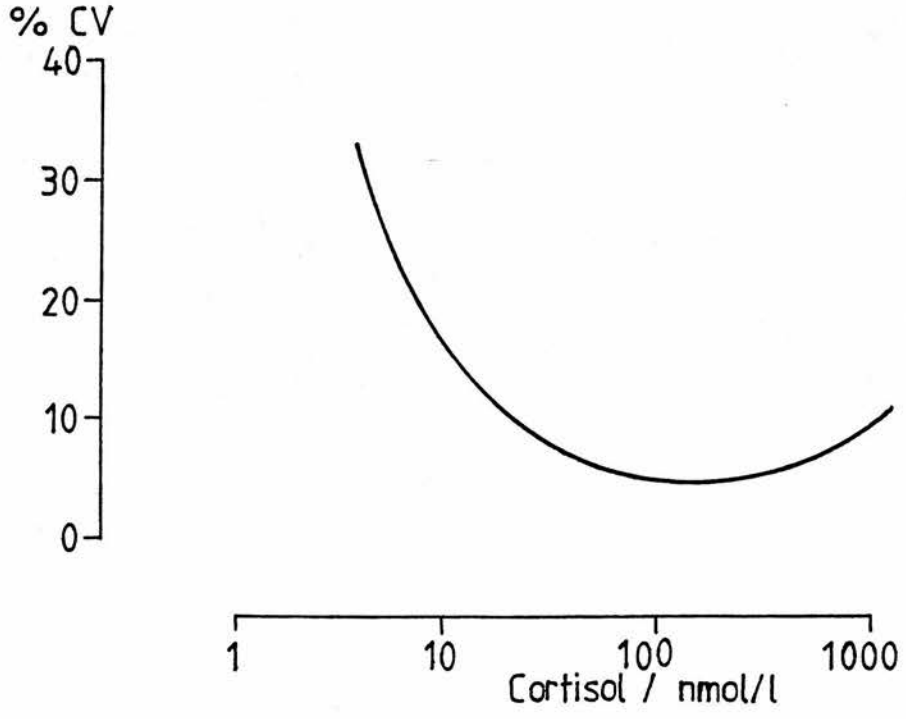

% CV
40
30
20
10
0
1
10
100
1000
Cortisol / nmol/l

FIGURE 2.16 Typical dose-response curve and precision profile for the sensitive cortisol RIA, as calculated by the WHO immunoassay program.

The inter-assay CV of the assays was assessed from values obtained for controls in several different batches (Table 2.6). The "in-house" method produces results with acceptable CV i.e. less than 10%, for cortisol concentrations 100-2000nmol/l. The sensitive assay was acceptable from 14-800nmol/l.

(b) Corticosterone Radioimmunoassay

Corticosterone antiserum and corticosterone stock in ethanol (approx. 3mmol/l) were kindly provided by Dr. B.C. Williams, Department of Medicine, Western General Hospital, Edinburgh. Corticosterone standards were prepared which covered the range 0.5-200nmol/l and were stored at -20°C. [^{125}I]-corticosterone was obtained from Immunodiagnostics Ltd. (Washington, Tyne & Wear).

The assay was performed essentially according to the method of Al-Dujaili, et al, (1981). In this assay a "pre-mix" was prepared which contained antibody (1:50 000) and [^{125}I]-corticosterone (427Bq/ml) diluted in 50mM phosphate buffer (pH 7.4; Appendix II). Sample (25µl) and "pre-mix" (200µl) were incubated overnight at 4°C. Following incubation, 500µl of charcoal suspension (Appendix II) were added to each tube and, after vortexing, the tubes were centrifuged (1700 x g; 30 min) and the supernatant

TABLE 2.6

CONTROL VALUES FOR STEROID RADIOIMMUNOASSAYS

ASSAY	No. of assays	CONTROL VALUES MEAN (nmol/l)	SD	CV (%)
CORTISOL:				
In-house method	5	212	18	8
	5	393	36	9
	5	773	43	5
Sensitive method	4	13	3	23
	4	80	9	11
	4	636	91	7
CORTICOSTERONE	8	1.1	0.1	9
	8	7.2	0.1	10
	8	82	17	21
ALDOSTERONE	8	0.59	0.07	12
	8	8.7	1.1	13

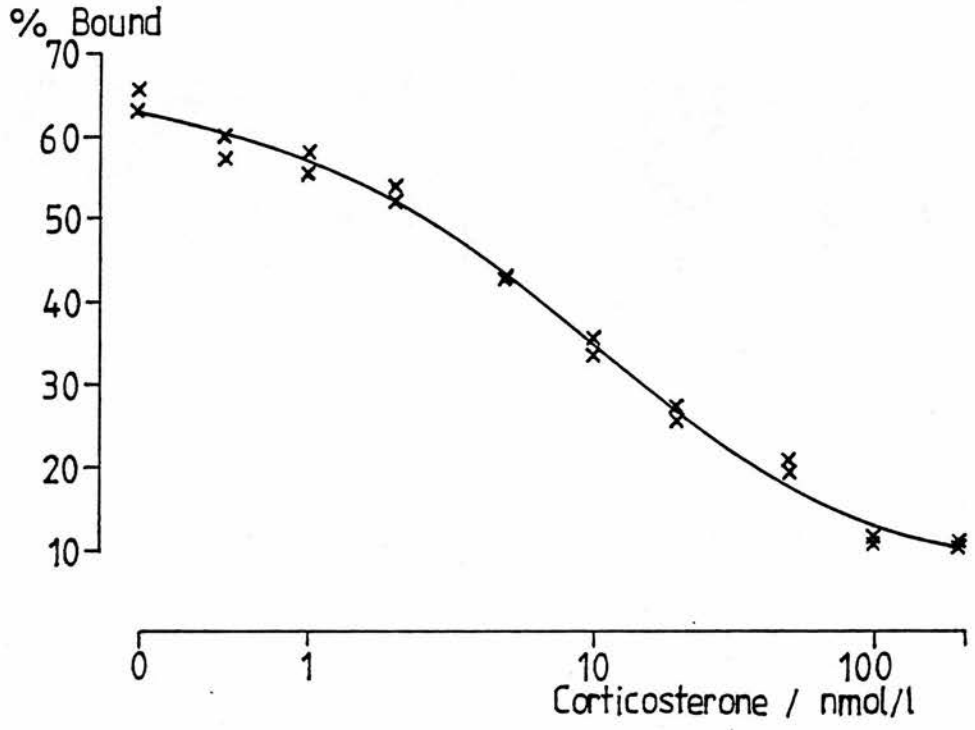
decanted. In this method it is the free [^{125}I]-corticosterone which is bound to the charcoal and counted.

Figure 2.17 shows a typical dose-response curve and precision profile for the corticosterone assay. The precision in the corticosterone assay was not as good as that in the cortisol assays. The assay was most reliable in the range 1.1-46nmol/l. The inter-assay CV (see Table 2.6) was approximately 10% which indicates that the precision between assays was better than that within assays.

(c) Aldosterone Radioimmunoassay

Antiserum against aldosterone was provided by Dr. B.C. Williams. Aldosterone stock solution in ethanol was freshly prepared from crystalline aldosterone obtained from Sigma Chemical Co. Ltd. (Poole, Dorset). Standards were prepared from this stock (approx. 3mM) by dilution in 0.2%BSA-EBS and covered the range 0.05-20nmol/l. These standards and the ethanol stock solution were stored at -20^{o}C. [^{125}I]-aldosterone was supplied by Immunodiagnostics Ltd. (Washington, Tyne & Wear).

The aldosterone assay method was based on that of Al-Dujaili and Edwards (1978). The method for the aldosterone assay was identical to the method for the


% Bound
70
60
50
40
30
20
10
0
1
10
100
Corticosterone / nmol/l

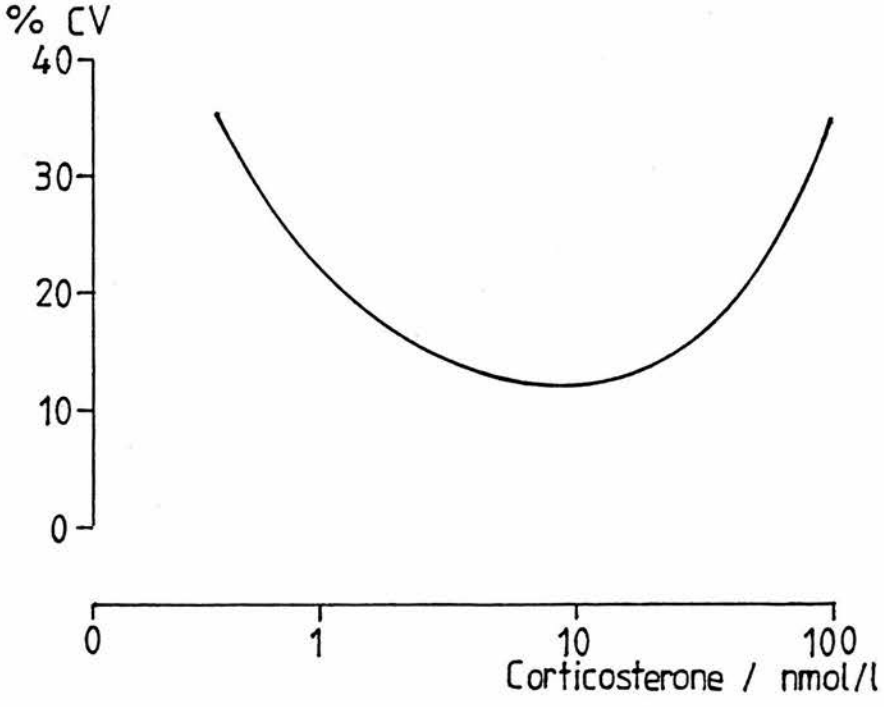

% CV
40
30
20
10
0
0
1
10
100
Corticosterone / nmol/l

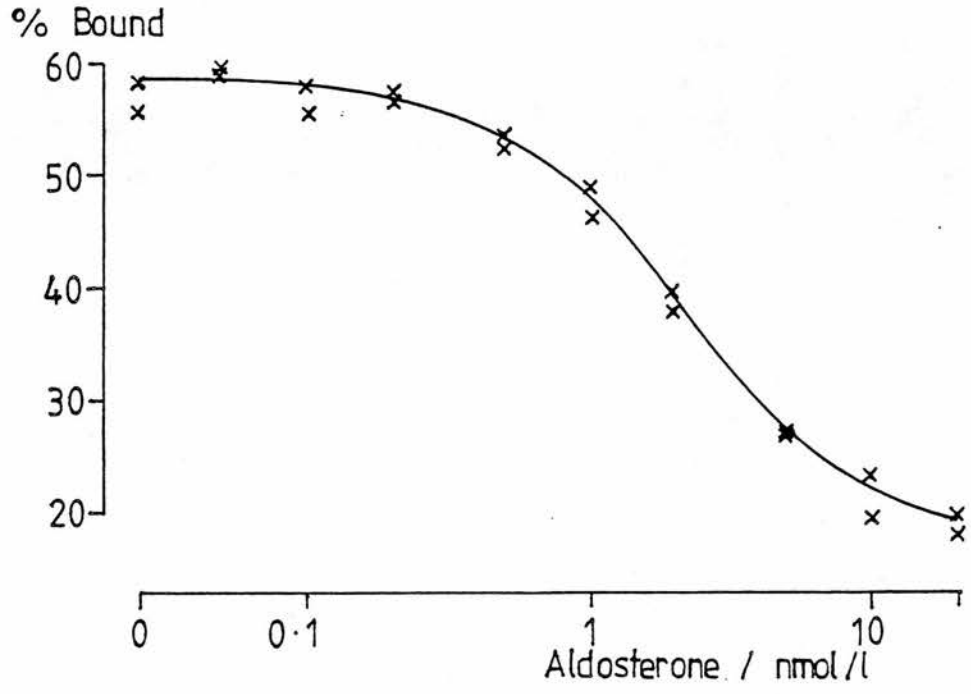
FIGURE 2.17 Typical dose-response curve and precision profile for the corticosterone RIA, as calculated by the WHO immunoassay program.

corticosterone assay except that the antibody was used at a 1:100 000 dilution and the [^{125}I]-aldosterone concentration was 444Bq/ml.

A typical dose-response curve and precision profile is shown in Figure 2.18. As with the corticosterone assay, the inter-assay CV (Table 2.6) was slightly better than the intra-assay CV.

8. MEASUREMENT OF NON-ESTERIFIED FATTY ACIDS USING THE COBAS BIO

Non-esterified fatty acids (NEFA) were measured using a colorimetric kit method (Wako NEFA C test kit) which had been adapted for use on a Cobas Bio centrifugal analyser following the procedure of Knox and Jones (1984). The Cobas Bio analyser is a less sophisticated version of the Cobas FARA. The Cobas FARA has more complicated but flexible software than the Cobas Bio but the optical and measurement systems are identical. The NEFA assay was an enzyme-linked method which produced a coloured end point. The absorbance was read at 550nm and NEFA concentrations in samples were calculated by comparison with known concentrations of standards. In this method the standards were dilutions of oleic acid, however, the kit is not specific for oleic acid nor is it intended to be. According to recovery results quoted in the manufacturer's


% Bound
60
50
40
30
20
0
0·1
1
10
Aldosterone / nmol/l

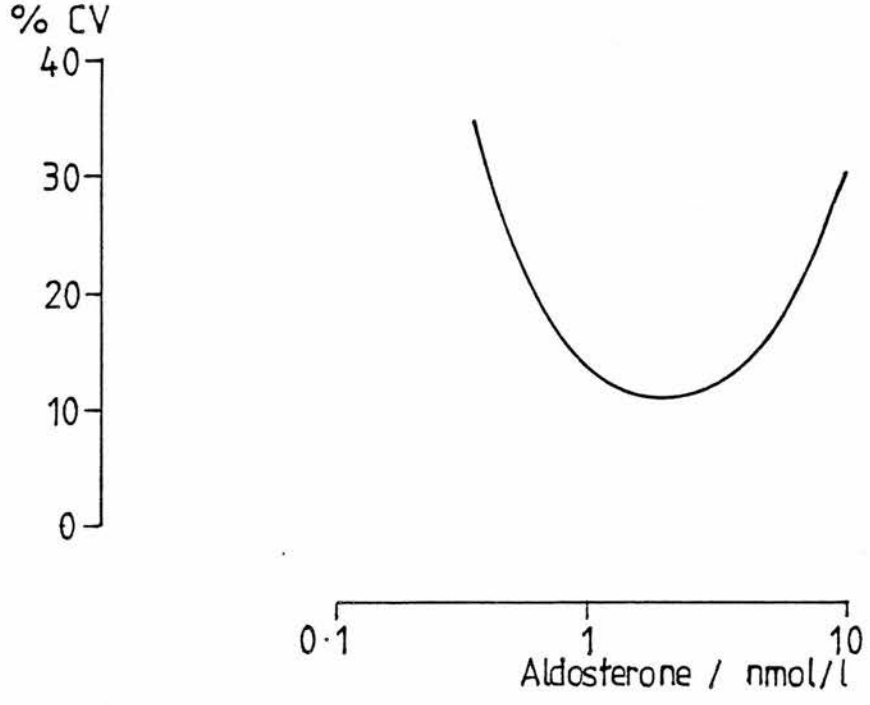

% CV
40
30
20
10
0
0·1
1
10
Aldosterone / nmol/l

FIGURE 2.18 Typical dose-response curve and precision profile for the aldosterone RIA, as calculated by the WHO immunoassay program.

instruction leaflet, the kit can detect both saturated and unsaturated NEFA's containing 6 to 22 carbon atoms.

9. GAMMA COUNTING

The radioimmunoassays were counted using an NE1600 gamma counter (Nuclear Enterprises, Reading, U.K.) linked to an Apple II computer. Standard curves were calculated from the raw counts using the WHO Immunoassay program (Version 5.3). This program was also used to interpolate values for the samples from the curves and to provide precision profiles for the assays.

10. TRYPTIC DIGESTION

Fractions from HPLC which showed both inhibitory activity and cross-reactivity with digoxin antibodies were pooled and subjected to tryptic digestion. Pooled fractions (200µl) were incubated with 20µl of trypsin (1g/l final concentration) at 37°C. The incubation was stopped after four hours by adding 20µl soya bean trypsin inhibitor (1g/l final concentration). The HPLC fractions were reconstituted in ammonium acetate (20mM, pH7.4), so controls for the tryptic digestion contained 200µl of this buffer in place of the fractions. After tryptic digestion, the samples and controls were measured for the ability to inhibit Na^+K^+ATPase in the enzyme-linked assay

on the Cobas FARA centrifugal analyser. Pooled fractions which had not been subjected to tryptic digestion were also assayed simultaneously with the samples and controls. Control incubates had no effect on the assay.

SECTION THREE

RESULTS

1. MEASUREMENT OF Na^+K^+ATPase INHIBITION AND DIGOXIN CROSS-REACTIVITY IN EXTRACTS OF HUMAN PLASMA

Plasma samples from normotensive and hypertensive subjects were stored at -20°C until required for assay. They were then thawed and extracted using Sep-Pak C_{18} cartridges. A new cartridge was used for each sample. Eluates were compared using both the colorimetric Na^+K^+ATPase assay and the Cobas FARA Na^+K^+ATPase assay.

(a) Colorimetric Na^+K^+ATPase Assay

(i) Pilot study

A pilot study was organised to assess whether measurable Na^+K^+ATPase inhibition could be extracted from the volume of plasma usually obtained from an individual patient. Plasma collected from 12 normotensive and 12 hypertensive patients was used for this study.

Eluates were dried down under air in a water bath at 50°C, reconstituted in non-adsorbed filtrate (350µl NAF) and stored at -20°C until assayed. The enzyme concentration in the Na^+K^+ATPase assay was 75U/l and the incubation time was 60min. Samples were assayed in triplicate with their own triplicate control for non-enzymic hydrolysis of ATP. In this control the incubation mixture was identical but the enzyme was added after the 1ml of 10% TCA.

Incubation mixtures which contained eluate produced significantly less phosphate than those containing NAF (Table 3.1). This indicated that it was possible to extract and concentrate inhibitory substances from the volume of plasma routinely obtained from a single patient. There was no significant difference between normotensives and hypertensives as assessed by a student's T-Test.

This pilot study highlighted a problem associated with reconstituting the eluate in NAF. The A_{700} values for the eluate were greater than those of the incubation control. Since the incubation control contained buffer in place of sample, this result indicated that a substance or substances present in NAF were interfering with the assay in some way. For this reason NAF was included in the reaction mixtures of the incubation control and the ouabain standards during the subsequent large study of samples from normotensive and hypertensive patients.

(ii) Large study

The large study comprised plasma samples from 32 normotensives and from 46 hypertensives. The range of ages and mean arterial blood pressures (MAP: diastolic + 1/3 pulse pressure) for normotensives and hypertensives were 26-57yrs and 27-65yrs and 87-107mmHg and 113-183mmHg respectively.

TABLE 3.1

INHIBITION OF Na^+K^+ATPase BY SEP-PAK ELUATES

	No. of samples	Phosphate produced in incubation mixture (nmol/l)
NORMOTENSIVES		
NAF	12	2.63×10^{-5}
Eluate	12	2.50×10^{-5} *
HYPERTENSIVES		
NAF	12	3.06×10^{-5}
Eluate	12	2.92×10^{-5} *

* $p<0.05$ with respect to NAF (Paired T-Test)

In this study the drying time was reduced by decreasing the methanol elution volume to 3ml. Eluates were reconstituted in 500µl of NAF, divided into 7 portions; 6 x 50µl, 1 x 200µl, and stored at -20°C. The 50µl portions were used in the Na^+K^+ATPase assay and the 200µl of eluate were retained for measurement of cross-reactivity in a digoxin RIA.

In contrast with the results in the pilot study, no significant difference was observed between the A_{700} of the incubations containing eluate and those containing NAF. This could have been due to the different reconstitution volumes used in the two assays; 350µl in the pilot study and 500µl in the larger study. A larger volume of eluate was required for the second study because eluates were assayed for cross-reactivity with digoxin antibodies as well as inhibitory capacity. This meant that the eluates in the large study were not concentrated to the same extent as those in the pilot study and so the concentration of the inhibitory substance may not have been high enough to be measurable with this assay. Another possibility was that the substances present in the NAF which increase the A_{700} of the incubation mixture may have masked the small amount of inhibition that was present.

The cross-reactivity of the eluates and NAF with digoxin antibodies was measured using a digoxin RIA kit obtained

from NEN, Massachusetts, U.S.A.. No significant difference was noted between the eluates from normotensive and hypertensive plasma and no correlation was observed between MAP and digoxin equivalent of the eluate.

(b) Enzyme-linked Na^+K^+ATPase Assay on Cobas FARA

The inhibitory capacity of eluates prepared from the plasma of a large number of normotensives and hypertensives was measured using the enzyme-linked Na^+K^+ATPase assay. Samples from many of the individuals in this study had also been assessed in the large study with the colorimetric Na^+K^+ATPase assay. 34 normotensives, age 27-57yrs, MAP 88-107mmHg, and 50 hypertensives, age 21-67yrs, MAP 113-173mmHg, were studied.

Some changes were made to the extraction protocol for this study. The Sep-Pak C_{18} extraction procedure was altered by increasing the amount of distilled water used to wash the cartridge to 10ml to ensure that all inorganic salts were being washed out of the cartridge before the sample was eluted. The drying temperature was also reduced to 37°C to prevent any possible loss of activity due to incubation at 50°C. Eluates were reconstituted in 170µl digoxin RIA buffer which eliminated the problems associated with reconstitution in NAF and made it possible to obtain quantitatively reliable results from the digoxin RIA.

Samples were thawed, extracted and assayed on the same day.

In all samples the NAF and the water wash were devoid of inhibitory activity. Inhibition due to eluates was converted to ouabain equivalents by interpolating values from a ouabain standard dose-response curve. This ouabain equivalent was then corrected for the concentration factor to give μmol/l ouabain in the original plasma. Cross-reactivity was quantified in digoxin equivalents which were similarly corrected to give pmol/l digoxin in the original plasma.

The mean ouabain equivalents and mean digoxin equivalents for the eluates prepared from the plasma of normotensive and hypertensive patients are shown in Table 3.2. These results were analysed using a Mann-Whitney U-test and there was no significant difference between the two patient groups. The dot diagrams in Figure 3.1 demonstrate the considerable variation in results. Linear regression analysis showed no correlation between MAP and ouabain equivalents, between MAP and digoxin equivalent or between ouabain equivalent and digoxin equivalent.

TABLE 3.2

OUABAIN AND DIGOXIN EQUIVALENTS PRESENT IN ELUATES FROM NORMOTENSIVE AND HYPERTENSIVE PLASMA

		NORMOTENSIVE SUBJECTS		HYPERTENSIVE SUBJECTS	
Concentration in original plasma sample		MEAN	SD	MEAN	SD
Ouabain equivalent:	µmol/l	0.61	0.31	0.59	0.78
Digoxin equivalent:	pmol/l	42.9	8.2	42.5	15.9
No. of subjects		34		50	

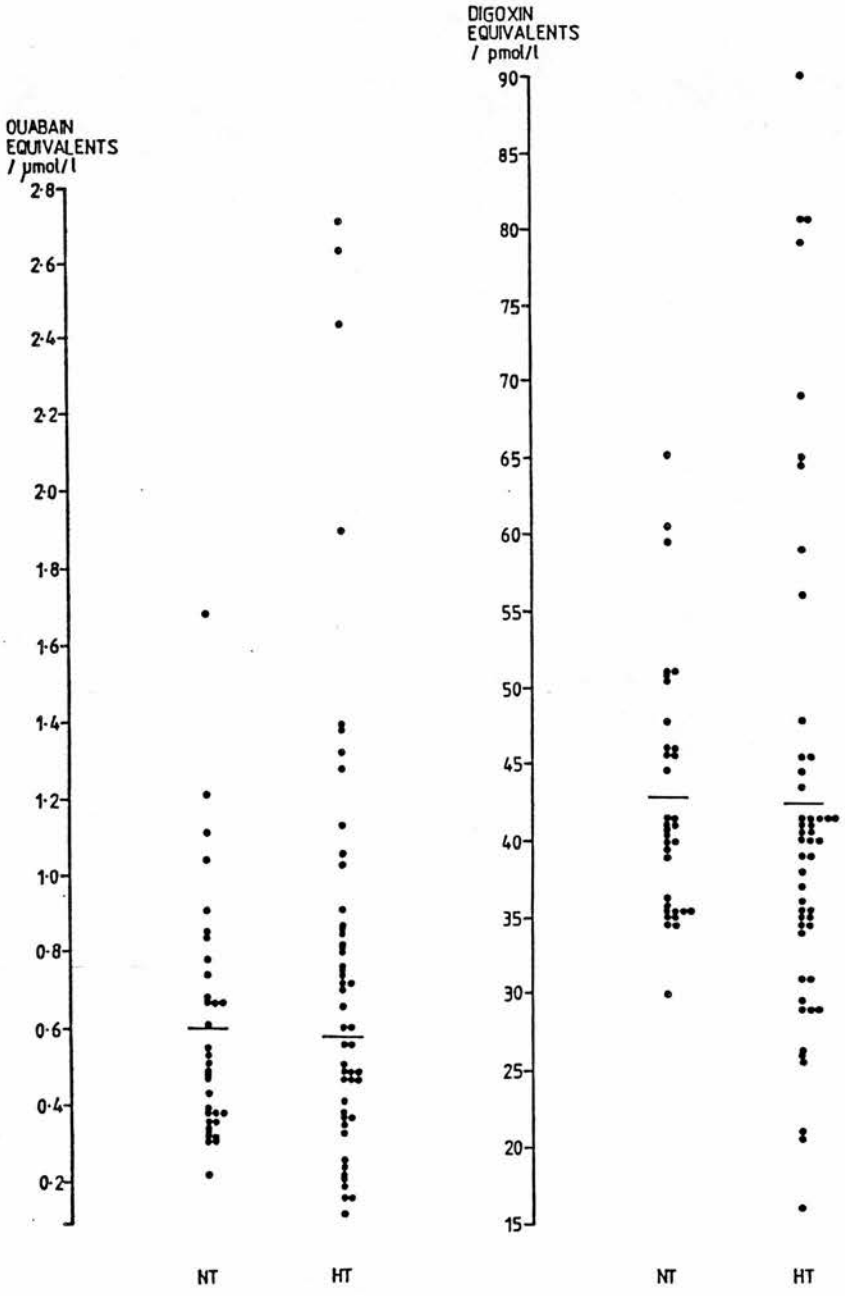

OUABAIN
EQUIVALENTS
/ µmol/l
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
NT
HT
DIGOXIN
EQUIVALENTS
/ pmol/l
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
NT
HT

FIGURE 3.1 Activity extracted from plasma samples obtained from normotensive and hypertensive subjects expressed as ouabain and digoxin equivalents (concentration in the original plasma sample). There was no correlation between these two activities.

2. MEASUREMENT OF Na^+K^+ATPase INHIBITION AND DIGOXIN CROSS-REACTIVITY IN CONCENTRATED ELUATES

(a) Concentrated eluates prepared from human serum

Pooled normal plasma and pooled polycythaemic serum and plasma were used to prepare sufficient eluate for dose-response curves of Na^+K^+ATPase inhibition. Details of the different concentrations achieved and the Na^+K^+ATPase assay conditions are given in Table 3.3. Concentrated eluates were reconstituted and diluted in NAF and assayed for the ability to inhibit Na^+K^+ATPase. As shown in Figure 3.2, only the concentrated eluate from the normotensive volunteers produced inhibition. No inhibition was observed with the concentrated eluate from the plasma of polycythaemic patients despite achieving a concentration factor which was twice that of the normotensive pool.

(b) Concentrated eluate prepared from bovine serum

Bovine serum was investigated as a potential source of Na^+K^+ATPase inhibitor because it was available in very large quantities from a local abattoir and could therefore be concentrated to a greater extent than human serum.

Blood was collected into plastic vessels and serum separated by centrifugation. Dilutions of concentrated

TABLE 3.3

CONCENTRATED ELUATES: CONCENTRATION FACTOR ACHIEVED AND COLORIMETRIC ASSAY CONDITIONS

Source of Plasma/Serum	Concentration Factor	Na^+K^+ATPase Assay Conditions	
		Enzyme Concentration	Incubation Length
Normotensive volunteers	120	25 U/l	4 h
Pooled serum from polycythaemic subjects	160	6.25 U/l	16 h
Hypertensive subject with polycythaemia	344	25 U/l	4 h

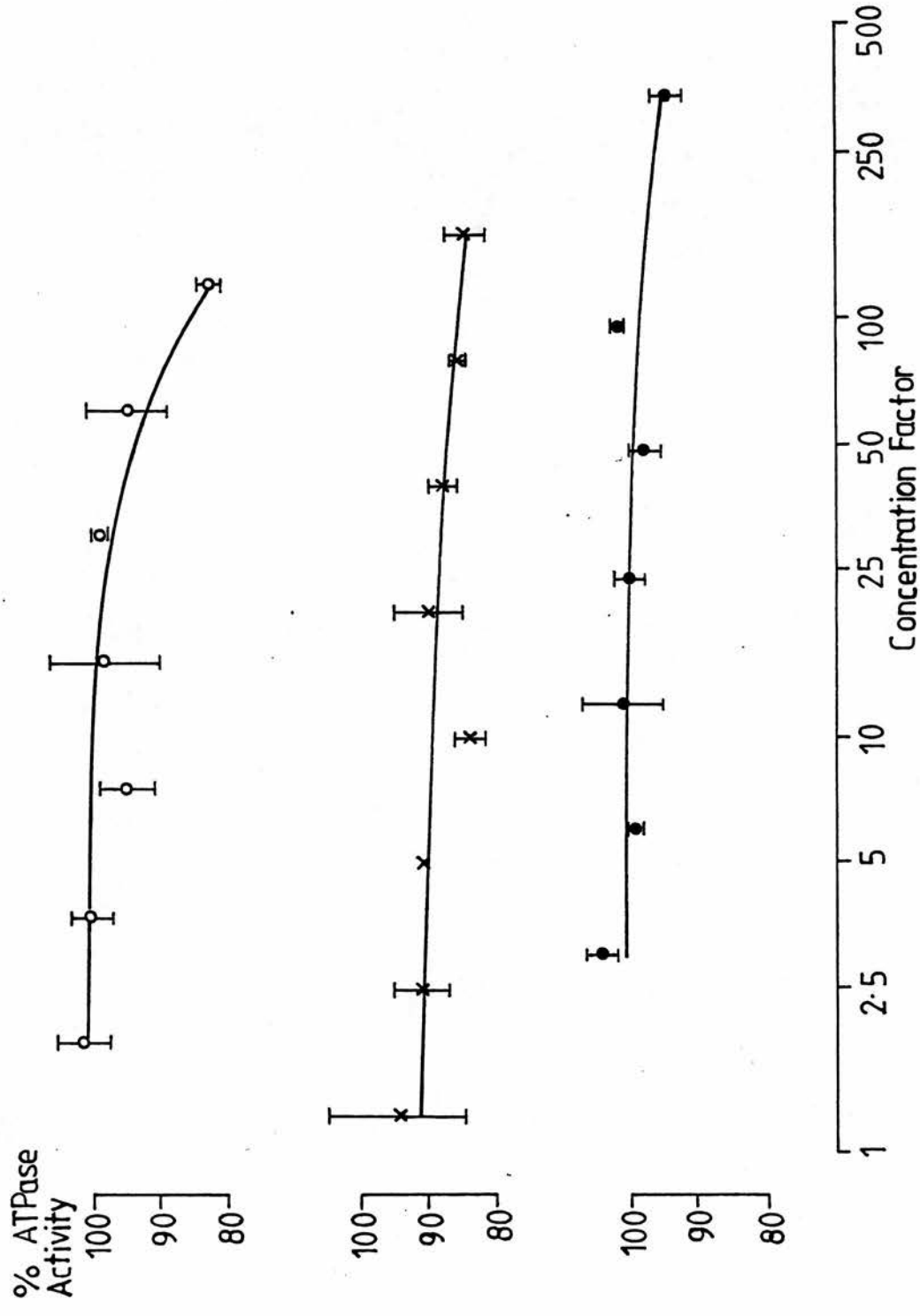

% ATPase Activity
100
90
80
100
90
80
100
90
80
1
2·5
5
10
25
50
100
250
500
Concentration Factor

FIGURE 3.2 Dose-response curves of concentrated eluates. Assay conditions are presented in table 3.3.

Eluate prepared from pooled plasma of normotensive subjects

Eluate prepared from pooled serum of polycythaemic subjects

Eluate prepared from plasma of a polycythaemic subject who was also hypertensive

eluate were tested for the ability to inhibit Na^+K^+ATPase and to cross-react with digoxin antibodies.

When dilutions of concentrated eluate were measured in the colorimetric Na^+K^+ATPase assay a curve was obtained with a maximum inhibition of 62% for a 200-fold concentration of serum (Fig.3.3).

Concentrated eluate in NAF was stored for nine months at -20°C. Once thawed it was divided into two aliquots; one was re-extracted on a Sep-Pak C_{18} cartridge and reconstituted in tris-buffered saline (TBS), the other aliquot remained in NAF. These eluates were diluted with the appropriate diluent; either NAF or TBS, and assayed on the enzyme-linked Na^+K^+ATPase assay on the Cobas Bio. The enzyme concentration was 250U/l, 10s readings were taken between 200s and 400s. Identical % inhibitions were obtained for the dilution curves in NAF in the colorimetric Na^+K^+ATPase assay and in the enzyme-linked assay but much more inhibition for a given concentration factor was obtained if the eluate was reconstituted in TBS (Fig.3.4). On further investigation it was shown that NAF contained approximately 20U/l of ATPase-like activity of which 15U/l was due to hydrolysis of ATP in the absence of Na^+K^+ATPase and 5U/l was due to ATP-independent oxidation of NADH. This indicated that the ability to detect low concentrations of inhibitor would be increased by

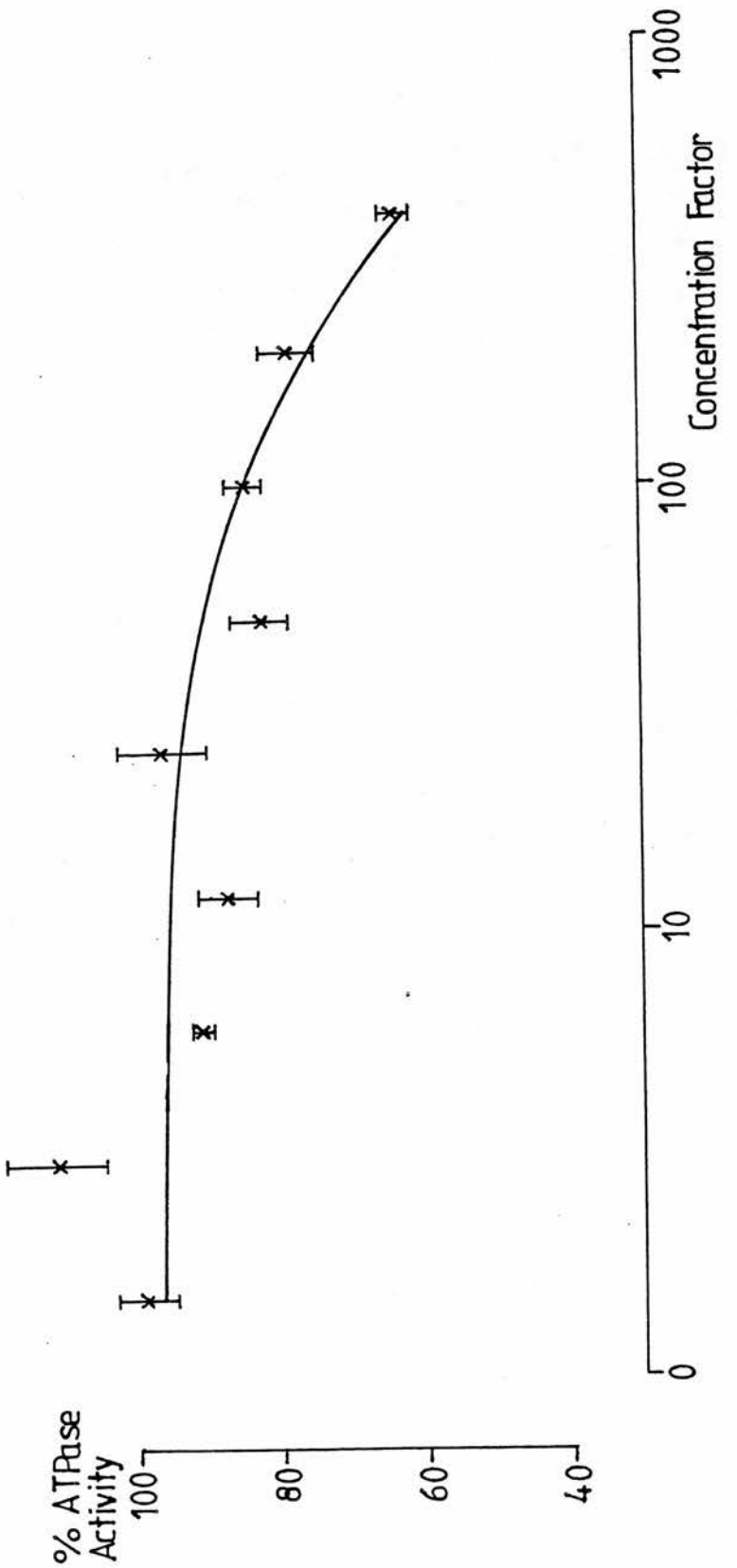
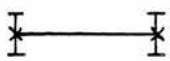

% ATPase Activity
100
80
60
40
0
10
100
1000
Concentration Factor

FIGURE 3.3 Dose-response curve of inhibition of Na^+K^+ATPase activity by concentrated eluate prepared from bovine serum measured using the colorimetric assay.



Mean and standard deviation of response (triplicate incubation)

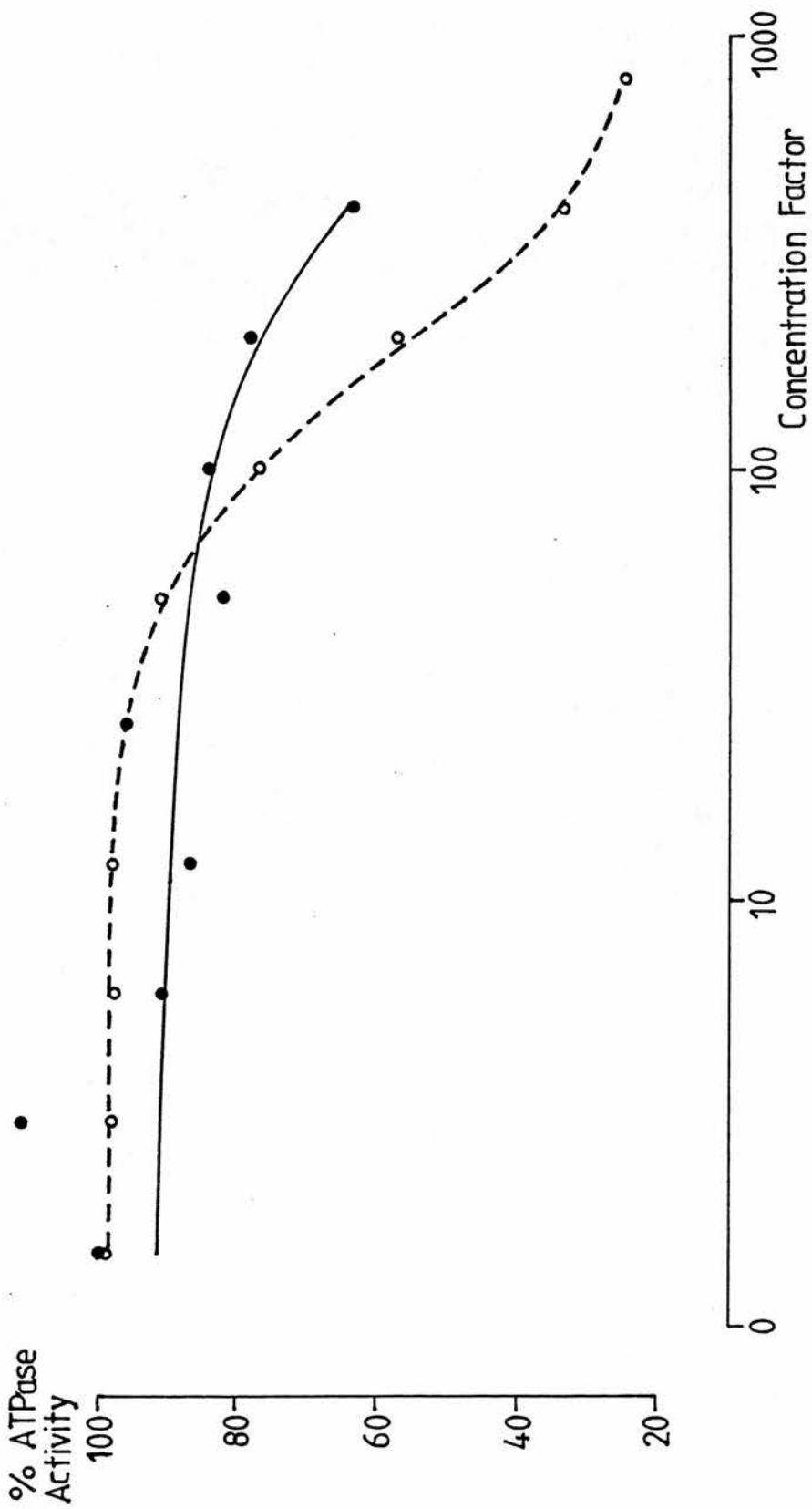

% ATPase Activity
100
80
60
40
20
0
10
100
1000
Concentration Factor

FIGURE 3.4 Dose-response curve of inhibition of Na^+K^+ATPase activity by concentrated eluate prepared from bovine serum measured using the enzyme-linked assay. Comparison of different diluents which shows that NAF contains substances which interfere in the assay and mask the inhibition.

●———● Diluent = NAF

o------o Diluent = Tris-buffered saline

reconstitution of eluates in buffer because there would appear to be something present in NAF which interferes in the Na^+K^+ATPase assay. This confirmed the earlier work with the colorimetric assay where it was observed that the presence of NAF in the incubation mixture resulted in a higher production of phosphate.

Concentrated eluate from bovine serum was diluted in NAF and the dilutions measured for the ability to cross-react in three different digoxin RIA's. Two commercial kits were used, a double antibody method from NEN and a coated tube method from Clinical Assays. An "in-house" method was also tested for cross-reactivity. This assay used SAPU reagents and was a pre-precipitated double antibody method. The bovine serum concentrated eluate cross-reacted in all three assays but to different degrees (Fig.3.5), probably because of variation in the affinity of the antibodies from the different assays for the cross-reactive substance(s). The NEN assay showed most cross-reactivity, the Clinical Assays method was least sensitive to the factor(s) in the eluate. The SAPU antibody showed adequate cross-reactivity and was used more frequently than the commercial assays for reasons of economy.

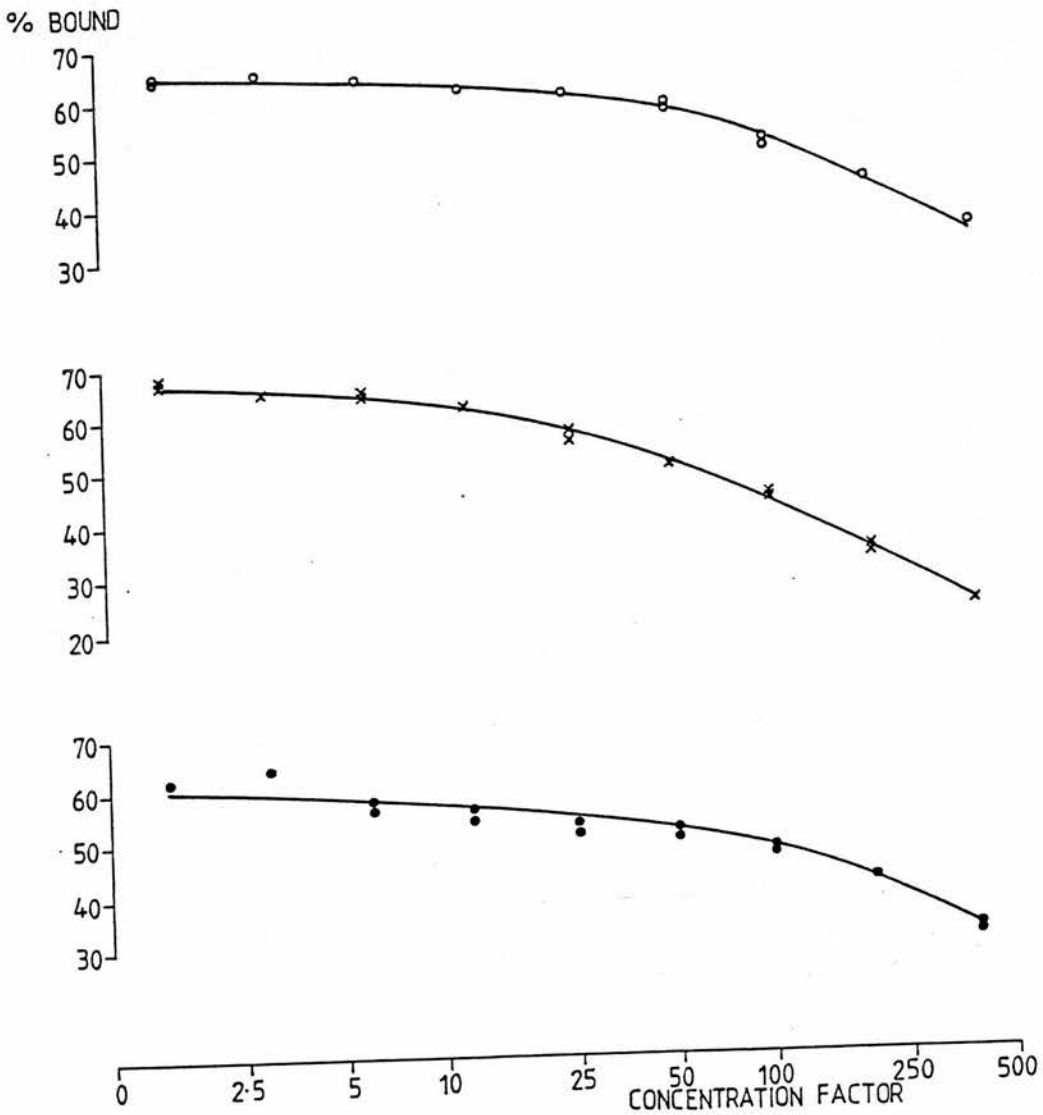
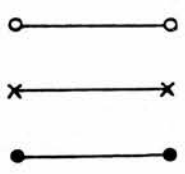

% BOUND
70
60
50
40
30
70
60
50
40
30
20
70
60
50
40
30
0
2·5
5
10
25
50
100
250
500
CONCENTRATION FACTOR

FIGURE 3.5 Cross-reactivity of concentrated eluate, prepared from bovine serum, in three different digoxin radioimmunoassays.



"In-house" method using SAPU antibody

Digoxin RIA kit supplied by NEN

Digoxin RIA kit supplied by Clinical Assays

3. SEPARATION OF ACTIVE FRACTIONS FROM BOVINE SERUM CONCENTRATED ELUATE USING HPLC

A second concentrated eluate was prepared from another pool of bovine serum and separated on high performance liquid chromatography (HPLC). A μBondapak C_{18} column was connected to a Waters gradient HPLC system and the sample eluted with a 20-100% acetonitrile gradient. Fractions were collected, dried down and reconstituted in ammonium acetate (pH7.4, 20mM) and measured for the ability to inhibit Na^+K^+ATPase and cross-react in a digoxin RIA.

Figure 3.6 shows the profile obtained when 1ml of eluate, which represented a 600-fold concentration of serum, was applied to the HPLC system. There were three peaks of inhibition and three peaks of digoxin cross-reactivity. Two peaks of inhibition co-eluted with two of the peaks of digoxin cross-reactivity but the major peak of inhibition did not cross-react with digoxin antibodies. The levels of inhibition at the summit of each peak were not very high. To achieve greater inhibition in each peak it would be necessary to concentrate the bovine serum to a greater extent. It was not possible to repeat this separation because there was insufficent sample.

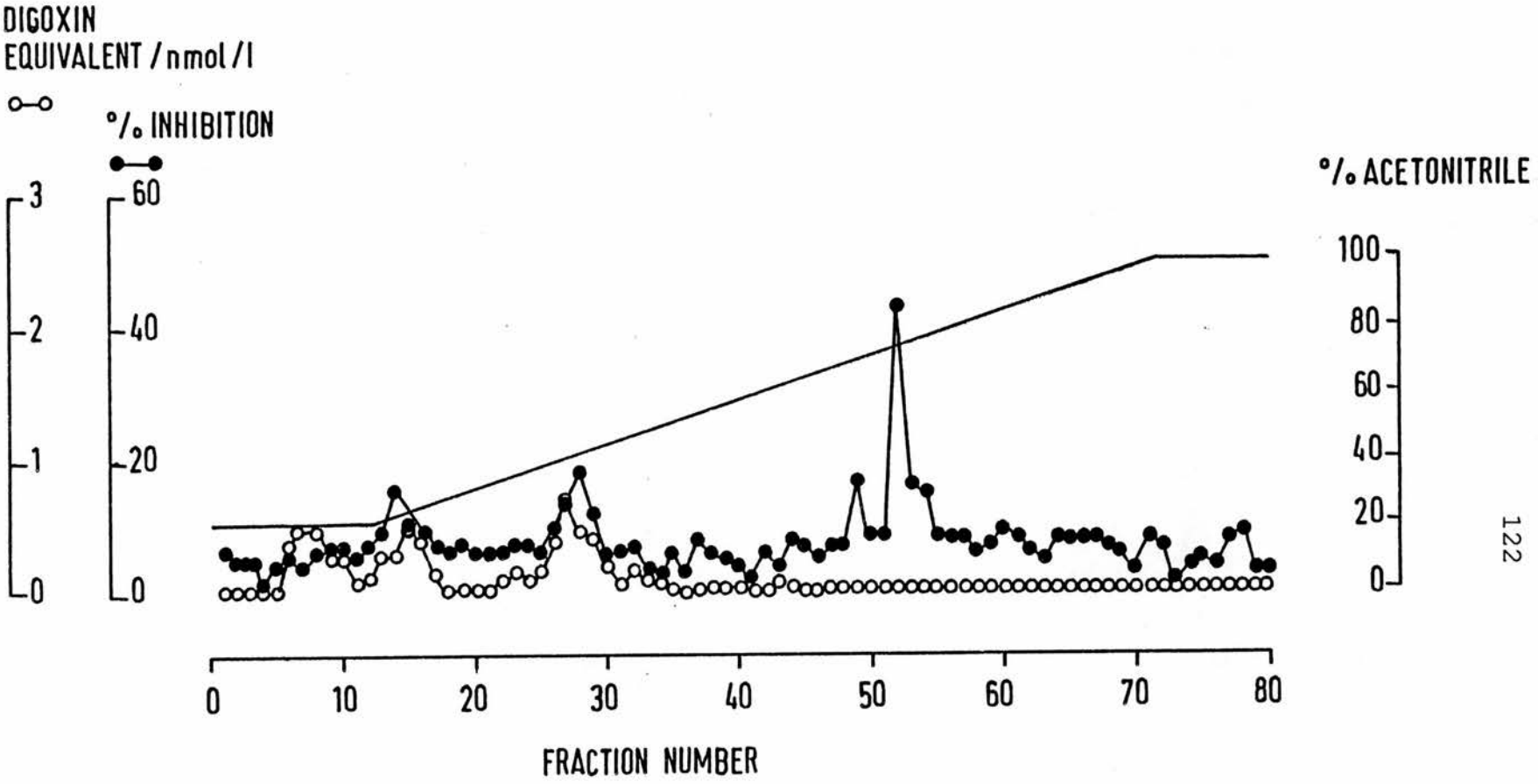

DIGOXIN
EQUIVALENT / nmol / l
% INHIBITION
% ACETONITRILE
3
2
1
0
60
40
20
0
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

FIGURE 3.6 HPLC profile of concentrated eluate prepared from bovine serum showing peaks of inhibitory activity and digoxin cross-reactivity. Sample = 1ml of 600-fold concentrated eluate (relative to original serum concentration).

●——● % Inhibition of Na^+K^+ATPase

o——o Cross-reactivity expressed as digoxin equivalents

4. EXTRACTION AND SEPARATION OF INHIBITION AND CROSS-REACTIVITY FROM BOVINE ADRENAL CORTEX

It was reported that digoxin-like cross-reactivity was present in the adrenal gland of the rabbit (Schreiber, et al, 1981b) and the rat (Schreiber, et al, 1981a; Valdes, et al, 1985). Since it was possible to extract inhibitory and cross-reactive substances from bovine serum, it was considered likely that the bovine adrenal gland could also be a source of these substances. Bovine adrenal cortex cytosol was extracted using Sep-Pak C_{18} cartridges and tested for the ability to cross-react with digoxin antibodies and inhibit Na^+K^+ATPase.

(a) Dilution curve of Sep-Pak eluate

Cytosol was prepared from bovine adrenal cortex and extracted on a Sep-Pak C_{18} cartridge to produce an eluate which was concentrated 200-fold, with respect to the original cytosol, in one step. The cytosol was shown to contain substances which were capable of inhibiting Na^+K^+ATPase and cross-reacting with digoxin antibodies in a dose-dependent manner (Fig.3.7).

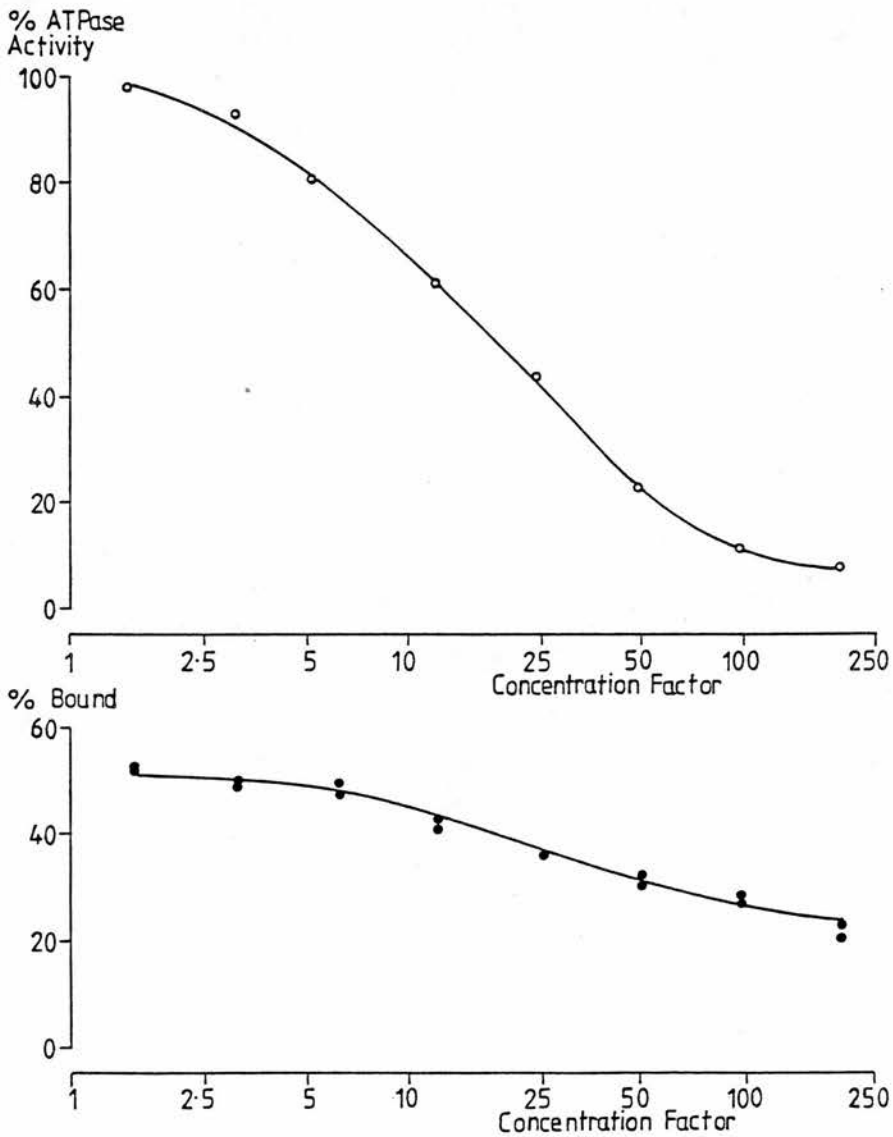
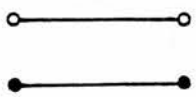
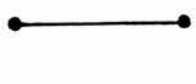

% ATPase Activity
100
80
60
40
20
0
1
2·5
5
10
25
50
100
250
Concentration Factor
% Bound
60
40
20
0
1
2·5
5
10
25
50
100
250
Concentration Factor

FIGURE 3.7 Dose-response curve of inhibition of Na^+K^+ATPase and cross-reactivity with digoxin antibodies by concentrated eluate prepared from adrenal cortex cytosol.



Inhibitory activity



Cross-reactivity

(b) Separation of active fractions from adrenal cortex cytosol eluate using HPLC

Adrenal cortex cytosol was concentrated using Sep-Pak C_{18} cartridges and the eluate was reconstituted in 20% acetonitrile to give a concentration factor of 200. The eluate was microfuged to remove particulate matter, injected onto a Waters μBondapak C_{18} column and eluted with a 20 to 100% acetonitrile gradient. 1ml fractions were collected, reconstituted in 250μl ammonium acetate (pH7.4, 20mM) and measured for the ability to inhibit Na^+K^+ATPase, cross-react with digoxin antibodies, cross-react with cortisol antibodies and for the presence of non-esterified fatty acids (NEFA).

HPLC of bovine adrenal cortex cytosol produced several peaks of Na^+K^+ATPase inhibition and several peaks of digoxin cross-reactivity but inhibition and cross-reactivity co-elute in only two positions (Fig.3.8).

Several peaks of cortisol cross-reactivity were also obtained which co-eluted with those peaks which cross-reacted with digoxin antibodies (Fig.3.9). The largest peak of cortisol cross-reactivity had a peak value of approximately 0.8mmol/l and was probably due to native cortisol.

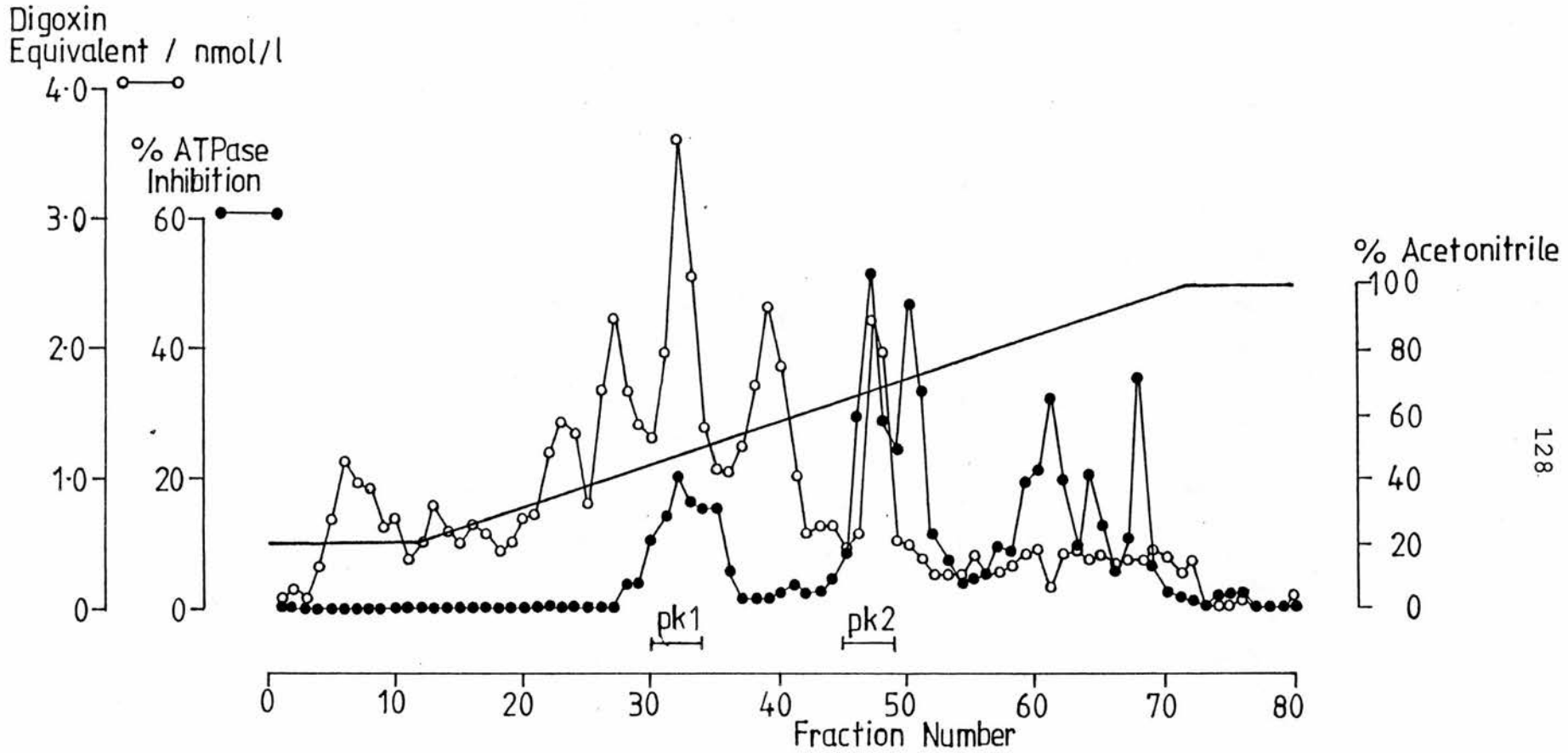
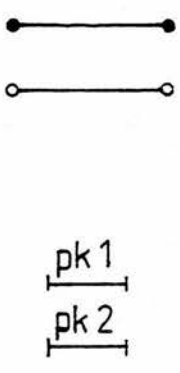

Digoxin
Equivalent / nmol/l
4·0
3·0
2·0
1·0
0
% ATPase
Inhibition
60
40
20
0
% Acetonitrile
100
80
60
40
20
0
pk1
pk2
0
10
20
30
40
50
60
70
80
Fraction Number

FIGURE 3.8 HPLC profile of concentrated eluate prepared from bovine adrenal cortex cytosol showing several peaks of inhibitory activity and several peaks of cross-reactivity. Sample = 1ml of 200-fold concentrated eluate (relative to original cytosol concentration)



●——● % Inhibition of Na^+K^+ATPase

o——o Cross-reactivity expressed as digoxin equivalents

pk 1, pk 2 Fractions pooled and tested for their effect on steroidogenesis

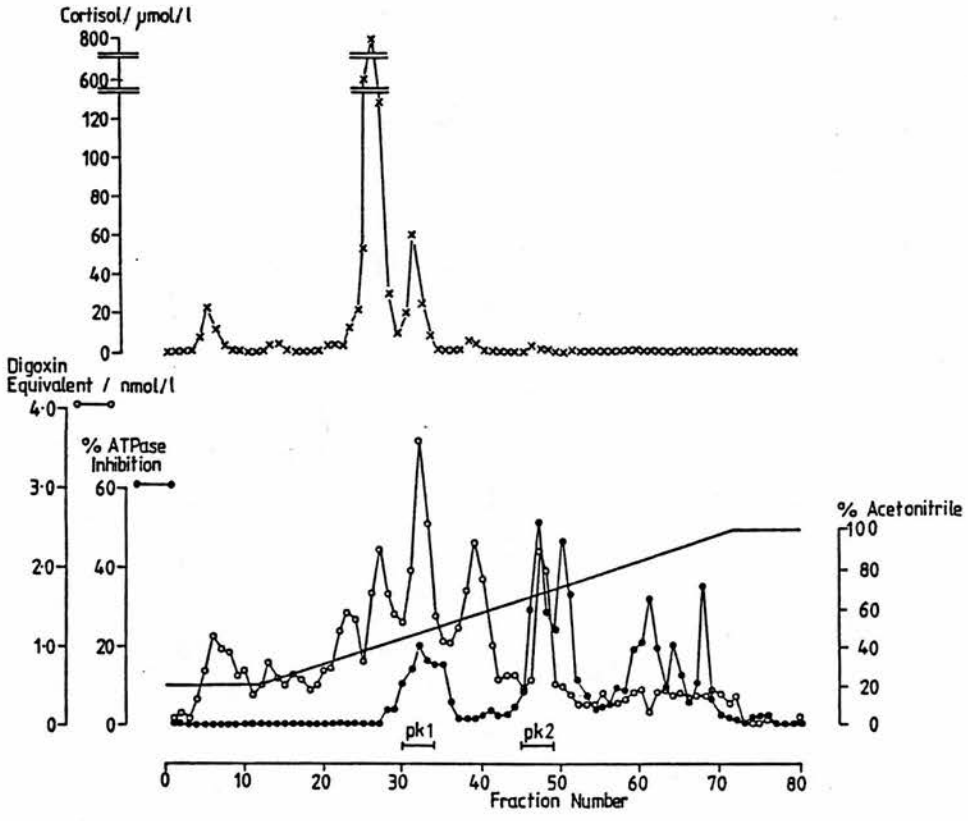

Cortisol/ µmol/l
800
600
120
100
80
60
40
20
0
Digoxin
Equivalent / nmol/l
4·0
3·0
2·0
1·0
0
% ATPase
Inhibition
60
40
20
0
% Acetonitrile
100
80
60
40
20
0
pk1
pk2
0
10
20
30
40
50
60
70
80
Fraction Number

FIGURE 3.9 HPLC profile of concentrated eluate prepared from bovine adrenal cortex cytosol showing elution position of peaks of cortisol cross-reactivity in relation to the peaks of inhibitory activity and digoxin cross-reactivity. Sample = 1ml of 200-fold concentrated eluate (relative to original cytosol concentration)

●——● % Inhibition of Na^+K^+ATPase

o——o Cross-reactivity with digoxin antibodies expressed as digoxin equivalents

×——× Cross-reactivity with cortisol antibodies expressed as μmol/l cortisol

NEFA was present in several fractions which eluted in four peaks before and one peak after those of interest (Fig.3.10). The last peak of NEFA co-eluted with a peak of inhibition but showed no cross-reactivity with the antibodies.

5. EFFECT OF ACTIVE FRACTIONS FROM HPLC OF BOVINE ADRENAL CORTEX ON STEROIDOGENESIS IN ISOLATED ADRENAL CELLS

The fractions from HPLC of bovine adrenal cortex cytosol which showed both inhibition and cross-reactivity were combined and designated peak 1 and peak 2 (see Fig.3.8). These peak fractions were tested on isolated adrenal cells for their effect on steroidogenesis.

Peak 1 had no effect on cortisol or corticosterone production in both glomerulosa cells and fasciculata cells but did stimulate aldosterone production in both cell types by 20-41 fold in the glomerulosa cells and 17-20 fold in the fasciculata cells.

The production of all three steroids was stimulated by peak 2. In glomerulosa cells there was a slight increase in cortisol production (as much as 2 fold) and a 17-20 and 20-49 fold increase in corticosterone and aldosterone production respectively. Cortisol production was

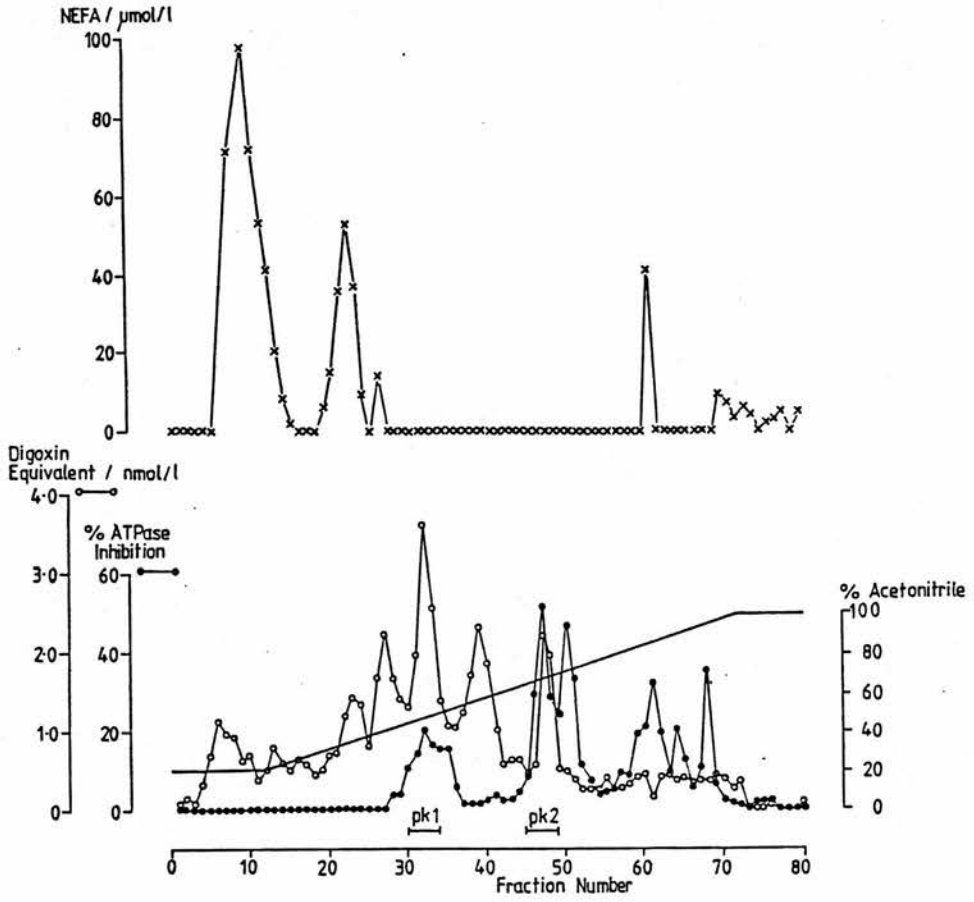

NEFA / µmol/l
100
80
60
40
20
0
Digoxin Equivalent / nmol/l
4·0
3·0
2·0
1·0
0
% ATPase Inhibition
60
40
20
0
% Acetonitrile
100
80
60
40
20
0
pk1
pk2
0
10
20
30
40
50
60
70
80
Fraction Number

FIGURE 3.10 HPLC profile of concentrated eluate prepared from bovine adrenal cortex cytosol showing the elution position of NEFA in relation to the peaks of inhibitory activity and digoxin cross-reactivity. Sample = 1ml of 200-fold concentrated eluate (relative to original cytosol concentration)

●——● % Inhibition of Na^+K^+ATPase

o——o Cross-reactivity with digoxin antibodies expressed as digoxin equivalents

×——× NEFA

stimulated 12-17 fold, corticosterone 10 fold and aldosterone 10-15 fold in fasciculata cells.

Where there was stimulation of steroid production it was far in excess of the stimulation which was observed with AII or ACTH; which achieved a maximal stimulation of 6 fold (Table 3.4). This may indicate that the stimulation due to the presence of the peak fractions arises because they are in fact steroid precursors. It is also possible that these are novel steroid stimulating compounds.

TABLE 3.4

STIMULATION OF BOVINE ADRENAL CORTEX CELLS BY ANGIOTENSIN II, ACTH AND HPLC FRACTIONS FROM ADRENAL CORTEX CYTOSOL

Stimulation of steroid production expressed as multiples of the basal steroid secretion of the cells.

Results from two cell preparations; A, B

AGONIST		GLOMERULOSA CELLS			FASCICULATA CELLS		
		CORTISOL	CORTICOSTERONE	ALDOSTERONE	CORTISOL	CORTICOSTERONE	ALDOSTERONE
ANGIOTENSIN II:	A	2	3	1	2	2	2
	B	1.7	2	2	3	4	2
	MEAN	2	2.5	1.5	2.5	3	2
ACTH:	A	1.5	3.4	1	2.5	2	1
	B	1.7	2	1.5	3	6	2
	MEAN	1.6	2.7	1.2	2.7	4	1.5
HPLC PEAK 1:	A	-	-	12	-	-	7
	B	-	-	41	1.5	-	17
	MEAN	-	-	26	0.7	-	12
HPLC PEAK 2:	A	2.5	10	25	10	10	8
	B	1.5	17	49	17	10	15
	MEAN	2	13	37	13	10	11

SECTION FOUR

DISCUSSION

It has been proposed (de Wardener, 1982) that essential hypertension develops as a result of a circulating natriuretic hormone which has its effect through the inhibition of Na^+K^+ATPase. Since cardiac glycosides are naturally occurring ligands for Na^+K^+ATPase, the natriuretic hormone has been sought on the basis that it may have similar properties and possibly structural similarities to the cardiac glycosides. Methods employed in the search for an endogenous cardiac glycoside-like substance have included measurement of ability to inhibit Na^+K^+ATPase, competition with [^{3}H]-ouabain for binding sites on Na^+K^+ATPase and cross-reactivity with digoxin antibodies or antibodies to other cardiac glycosides.

In this thesis, fractions extracted from plasma and cytosol were investigated for the possession of two of these properties; inhibition of Na^+K^+ATPase and cross-reactivity with digoxin antibodies. It was necessary to develop methods for measuring Na^+K^+ATPase and, although a sufficiently sensitive RIA for digoxin was in routine use in the Clinical Chemistry department, it was adapted for use with a non-serum matrix. An extraction procedure was developed and tested for its ability to extract cardiac glycoside-like activity from serum or plasma and from adrenal cortex cytosol. Extracts from adrenal cortex were tested for their effect on steroidogenesis in isolated cells.

1. PERFORMANCE OF METHODS

(a) Assays of cardiac glycoside-like activity

Different workers have used a variety of methods to measure the inhibition of Na^+K^+ATPase by serum or tissue extracts. The most direct assays of enzyme activity comprise an incubation followed by measurement of the phosphate released by the action of the enzyme on ATP. Several methods have been used to quantify the phosphate released; a colorimetric method (Gruber, et al, 1980), measurement of the ^{32}P released from [^{32}P]-ATP (Cloix, et al, 1983) and by an enzyme-linked method where the amount of phosphate produced by the Na^+K^+ATPase was quantified by the decrease in absorbance at 340nm due to the oxidation of NADH to NAD^+ (Hamlyn, et al, 1982). A cytochemical method has also been used to measure the inhibition of Na^+K^+ATPase. This less direct method depends on the relationship between the activities of two enzymes; stimulation of glucose-6-phosphate dehydrogenase activity has been shown to correlate with inhibition of Na^+K^+ATPase (Fenton, et al, 1982). Stimulation of glucose-6-phosphate dehydrogenase was quantified by the densitometric measurement of colour produced by the action of the enzyme from guinea pig kidney slices. The ability of serum or tissue extracts to inhibit the binding of [^3H]-ouabain to Na^+K^+ATPase has been assessed using intact red blood cells (Devynck, et al,

1983) and using partially purified enzyme (Kelly, et al, 1985).

In the studies presented in this thesis, samples were tested for the ability to inhibit Na^+K^+ATPase using two different, but direct, methods for measurement of enzyme activity. In the first, the phosphate released by the hydrolysis of ATP was measured by a colorimetric method, and in the second method the hydrolysis of ATP was linked to the oxidation of NADH by the enzymes lactate dehydrogenase and pyruvate kinase.

In the colorimetric assay, samples were incubated with the enzyme in triplicate with a single measurement of the phosphate released. Although the inter-assay CV had been assessed over six assays as 8%, it was noted that during the large study, comparing samples from normotensives and hypertensives, that the CV deteriorated to 14% (16 assays over a period of two months). Variability in the commercially prepared enzyme could have been a contributory factor, this will be discussed later. The colorimetric assay also depended on a 16h incubation of the enzyme which, with hindsight, was an ill-advised choice of incubation time. As it was unlikely that the enzyme was still functioning after this length of time and the stability of the Sep-Pak eluates was not known, it was an error of judgement to use such incubation conditions.

However, since controls were routinely included to measure non-enzymic hydrolysis of ATP, and each sample measured with this assay had its own triplicate control, the values obtained were still a measure of the activity of the enzyme. During the period when the colorimetric assay was in use the Sep-Pak eluate was always reconstituted in its own non-adsorbed filtrate (NAF). The intention was to dilute the eluate in a matrix with similar characteristics to plasma. For this reason the NAF was also measured in the colorimetric assay to ensure that any inhibition which was measured was due to the eluate and not substances present in the NAF. As discussed later it was noted that the NAF interfered in the assay in a manner which resulted in an increase in the absorbance readings so any inhibition which was measured was entirely due to the presence of the eluate.

In addition to the problems outlined above, the colorimetric assay was an extremely labour intensive method because it depended on so many steps and, since as many controls as samples were measured, it was only possible to measure the inhibitory capacity of approximately 12 samples per assay. The acquisition of a Cobas centrifugal analyser provided the oppportunity to develop an enzyme-linked assay which would permit the rapid analysis of a substantially greater number of samples. This was of particular importance when measuring the inhibitory

capacity of the 80 fractions from a single HPLC separation.

The precision was excellent in the enzyme-linked assay and the assay time was considerably reduced. This meant that reliable values for the inhibitory capacity of samples were obtained by a single measurement on each sample. By this method as many as 26 samples could be assayed in a single batch, which took approximately 10 minutes, compared with 12 samples in one and a half days for the colorimetric method. The concentrations of ouabain standards in the two assays indicated that the enzyme-linked assay was apparently less sensitive; colorimetric assay 0.005-25μmol/l, enzyme-linked assay 0.25-1000μmol/l. This was because a much higher Na^+K^+ATPase concentration was used in the enzyme-linked assay so the ratio of Na^+K^+ATPase to inhibitor was lower thus more inhibitor was required to inhibit the enzyme by the same amount. However, the better precision and the smaller sample volume required in the enzyme-linked assay compensated for this loss in sensitivity. The enzyme-linked assay was capable of detecting much smaller levels of inhibition. The inter-assay CV over six consecutive assays was 8% in the colorimetric assay compared with 1% in the enzyme-linked assay. The detection limits for the two assays were, therefore: 2% inhibition for the enzyme-linked assay and 16% for the colorimetric assay, equivalent to approximately

0.5 and 0.15μmol/l ouabain respectively. Detection limit was defined as the concentration of ouabain which inhibited the enzyme to a value which was two standard deviations more than that of the uninhibited enzyme. Since the enzyme-linked assay requires a much smaller sample (20μl for a single assay) than the colorimetric assay (300μl for triplicate assay and triplicate control), allowing greater concentration of the sample, and the dilution in the enzyme-linked assay was much less (20/250 compared with 50/1000), the difference in sensitivity becomes unimportant.

The Na^+K^+ATPase used in both the colorimetric assay and the enzyme-linked assay was a commercially prepared enzyme obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Variation in the activity of this enzyme could have contributed to the problems encountered in the colorimetric assay. Although many workers in this field use the same enzyme preparation it is possible to purify Na^+K^+ATPase which has a much higher specific activity by what appears to be a relatively simple method (Jørgensen, et al, 1974). By preparing the enzyme "in house" it would have been possible to greatly reduce the incubation time required in the colorimetric assay.

Although it has been demonstrated in this thesis that Sep-Pak eluates and HPLC fractions are capable of inhibiting

Na^+K^+ATPase in the enzyme-linked assay it has not been shown that the inhibition measured was due to a specific action on the Na^+K^+ATPase. The linking enzymes, pyruvate kinase and lactate dehydrogenase, were present at concentrations twenty times that of the Na^+K^+ATPase, however, if there was substantial inhibition of these enzymes it could have been misinterpreted as inhibition of the Na^+K^+ATPase.

If the substance of interest was truly acting on the Na^+K^+ATPase then if the sample was added to the reaction mixture in the presence of adenosine diphosphate and in the absence of Na^+K^+ATPase there should be no inhibition of the oxidation of NADH. Inhibition would be measured if the sample is acting by inhibiting one of the linking enzymes. It is also not known if the inhibition is reversible, an important point if the sample is to be of biological significance. Assuming that the substance acts in an analogous way to the cardiac glycosides this could be investigated by attempting to reverse the inhibition by increasing the potassium concentration.

(b) Radioimmunoassay for Digoxin

Sep-Pak eluates showed cross-reactivity with digoxin antibodies in both of the commercial digoxin RIA kits and in the "in-house" digoxin RIA. The "in-house" method was

used for the majority of the work contained in this thesis. It was shown that the sample matrix affected the results obtained with this assay (Fig.2.8, p65). When digoxin standards were diluted in ammonium acetate (20mM, pH7.4) or TTS, the buffer used in the enzyme-linked Na^+K^+ATPase assay, the curve was displaced relative to the curve obtained when standards were diluted in serum. Standards diluted in RIA buffer were chosen for routine use because they produced a curve which was similar to the serum curve (see Fig.2.8, p65).

Ammonium acetate was investigated as a possible standard diluent because HPLC fractions were reconstituted in ammonium acetate (20mM, pH7.4) in order that fractions could be pooled and concentrated by freeze drying, if required, since ammonium acetate would not be concentrated by the freeze drying whereas non-volatile buffer salts would be concentrated to levels at which they may interfere in the assays. Reconstitution of HPLC fractions in ammonium acetate meant that digoxin equivalents were not quantitatively accurate, because of the curve displacement, but since it was the elution profile which was of primary interest, and not the absolute value of the peak heights, this did not invalidate the results. However, in the second large study comparing normotensive and hypertensive subjects, eluates were reconstituted in RIA buffer to ensure that quantitatively accurate results were obtained.

(c) Sep-Pak Extraction

The Sep-Pak extraction method proved to be a simple and rapid method for the preparation of samples. Ions pass straight through the cartridges and, because the cartridges were subsequently washed with water to remove very polar substances, the final eluate should be free of ions. This was verified when the lithium concentration was measured in untreated plasma, the water wash and non-adsorbed filtrate (NAF), and all lithium was accounted for in the NAF. The concentration of metal ions in the sample would present a possible source of interference in any Na^+K^+ATPase assay and could result in artefactual findings. It has been suggested that heating samples to temperatures above 40^oC and acidification could also produce artefacts (Tal, et al, 1986). Sep-Pak extraction is a more gentle and specific method of extracting substances of interest than deproteinisation of samples by acidification and boiling. Using Sep-Pak cartidges, substances are extracted according to their polarity.

In early work on this thesis, Sep-Pak eluates were reconstituted in NAF in order to provide an internal control but it was observed (see p113) that when the bovine concentrated eluate dilution curve was prepared in buffer instead of NAF the inhibition obtained for a given concentration factor was greater. This suggested that the

NAF contained substances which interfered with the Na^+K^+ATPase and so masked the inhibition present in the sample. Following this observation all eluates were subsequently reconstituted in buffer in order to avoid this problem.

The recovery experiments using the Sep-Pak extraction method were less than satisfactory. A contributory factor was that recovery was assessed by the measurement of Na^+K^+ATPase inhibition. These experiments could be improved by using [^{3}H]-ouabain as the model and assesssing the recovery by liquid scintillation. Until the factor has been isolated, the measurement of recovery using ouabain can only provide an approximate guide to the recovery of the cardiac glycoside-like substance(s).

(d) High Performance Liquid Chromatography

The HPLC separation was extremely reproducible as assessed by the pattern of peaks obtained by chromatography of bovine adrenal cortex cytosol. One drawback of the method was that the only "handle" on the substances of interest was their inhibition of Na^+K^+ATPase and their cross-reactivity with digoxin antibodies. This meant that the result of a single HPLC separation was not known until the fractions had been dried, reconstituted in buffer and assayed, a process which took a minimum of 36 hours.

Consequently, it took longer to optimise the method because it was not possible to see the effect of altering chromatographic conditions immediately.

(e) Isolated adrenal cell preparation

Isolated zona glomerulosa and zona fasciculata cells were prepared from bovine adrenal glands and their responsiveness to AII and ACTH was measured. Both cell types were dose-responsive to AII and ACTH and the highest concentrations were chosen for inclusion as controls in the experiments on the effect of HPLC extracts on steroidogenesis. There was considerable difference in the size of the response between cells prepared on different days; 2 and 3-fold stimulation of steroidogenesis on one occasion, 2 and 6-fold stimulation on another. The difference between cell preparations could have been a consequence of events which occurred at the abattoir, for example, the delay between death and removal of the adrenal glands, and which were outwith my control.

(f) Radioimmunoassays for adrenal steroids

Two different separation methods were employed in the adrenal steroid RIA's; preprecipitated double antibody and charcoal separation. The two cortisol assays employed preprecipitated double antibody separation. Precision in

these assays was reasonable except at low concentrations in the sensitive assay where the low control (approx. 8nmol/l) had a higher CV (28%). In the aldosterone assay the control CV's had a mean of 12.5% and in the corticosterone assay the low and medium controls had CV's less than 10% but the high control had a CV of 21%. These values were acceptable but if these assays were to be used for a much longer study it would be desirable to attempt to improve the precision in these assays.

2. INVOLVEMENT OF ENDOGENOUS CARDIAC GLYCOSIDE-LIKE SUBSTANCES IN HYPERTENSION AND OTHER CONDITIONS

(a) Endogenous cardiac glycoside-like substances in conditions other than hypertension

In the natriuretic hormone hypothesis, (de Wardener and MacGregor, 1982), it is proposed that the stimulus for the release of the hormone is central volume expansion. Therefore, it is of interest that digoxin-like immunoreactivity (DLIR) and Na^+K^+ATPase inhibition have been measured in the serum of patients suffering from a variety of conditions related by a disturbance of fluid balance.

There have been many reports of the presence of DLIR in serum from neonates (Pudek, et al, 1983), and in serum

(Graves, et al, 1984) and amniotic fluid (Graves and Wiliams, 1984) of 3rd trimester pregnant women. Pudek, et al, (1983) also found no relationship between dehydroepiandrosterone sulphate (DHEA-SO_4) and DLIR, although others have reported that DHEA-SO_4 was responsible for some of the DLIR present in plasma (Vasdev, et al, 1985). It has been reported that the levels of cross-reactivity and inhibition in the amniotic fluid of third trimester pregnant women were significantly correlated with their diastolic blood pressure at time of amniocentesis and hypertensive pregnant women had significantly higher levels than normotensive women (Graves and Williams, 1984). This is supported by recent work (Gregoire, et al, 1988) in which a radioreceptor assay was used to measure a ouabain-displacing factor present in urine. Normal pregnancy significantly increased the levels of the factor and greater increases were measured in the urine of subjects who had pregnancy induced hypertension or pre-eclampsia. However, Goodlin (1987) reported that the digoxin cross-reactivity present in serum of women with pre-eclampsia was often not much higher than that in serum of normotensive pregnant women but if the pre-eclampsia was accompanied by liver or renal dysfunction or expanded toxaemic syndrome the serum levels of DLIR increased occasionally to values which were in the therapeutic range for digoxin. The difference between the results of Gregoire, et al, (1988) and Goodlin (1987) could be because they are using

different assays or because they were investigating different body fluids.

Raised levels of digoxin cross-reactivity have also been reported in patients with liver disease (Greenway and Nanji, 1985; Nanji and Greenway, 1985) and different values for the DLIR content of a sample were obtained with different digoxin assay kits (Greenway and Nanji, 1985). Higher levels of DLIR were reported when liver disease was accompanied by ascites and oedema (Nanji and Greenway, 1986) which could be related to the volume expansion associated with these conditions.

It has been suggested that the hyponatraemia which often follows aneurysmal subarachnoid haemorrhage could be due to the release of a digoxin-like natriuretic factor, possibly as a result of hypothalamic damage (Wijdicks, et al, 1987).

It was shown that plasma volume was expanded in patients with acromegaly (Deray, et al, 1987), a condition in which there is excess production of growth hormone, and that there was a significant correlation between plasma volume and the capacity of serum from these patients to inhibit Na^+K^+ATPase.

It has been known for some time that there are sodium transport abnormalities associated with uraemia (Cole,

1973; Edmondson, et al, 1975a) and that uraemic serum contained a factor which inhibited sodium efflux in erythrocytes (Kramer, et al, 1976) and inhibited brain Na^+K^+ATPase (Minkoff, et al, 1972). It was subsequently reported that falsely raised (Belpaire, 1975) or false positive (Graves, et al, 1983) digoxin levels were measured in patients with renal failure. Graves, et al, (1983) reported that as many as 60% of patients had false positive values, however, Greenway and Nanji (1986) found only a 23% false positive rate which could be due to batch-to-batch differences in antibody since some kits were obtained from the same manufacturers as Graves, et al.

The activity of erythrocyte Na^+K^+ATPase in patients with renal failure was asessed by Izumo, et al, (1984) using a ^{86}Rb uptake method. In this method, the active transport of [^{86}Rb] into the cell reflects potassium transport by the enzyme and thereby provided a measure of the Na^+K^+ATPase activity. Dialysis improved the activity of the enzyme, which was 30% of the control values before dialysis, and incubation of normal cells in pre- and post-dialysis uraemic serum demonstrated that the inhibition of the enzyme was caused by a humoral factor. Izumo, et al, (1984) reported no difference in the number of pump sites, as assessed by [^3H]-ouabain binding, but Cheng, et al, (1984) suggested that a decrease in the number of pump sites was responsible for the high erythrocyte

intracellular sodium measured in 16 of the 45 end stage renal patients on dialysis that they studied.

Early reports of transport abnormalities in erythrocytes of renal failure patients could be explained by the presence of Na^+K^+ATPase inhibitors in their serum. Studies of the effect of dialysis on the levels of these inhibitors have produced disparate results. Kelly, et al, (1986a) report that the amount of inhibition increased acutely during dialysis and that chronic dialysis returned values to the normal range but Deray, et al, (1986), report that dialysis acutely decreased the inhibitory activity in serum of renal failure patients to levels found in normotensive control patients.

The presence of cardiac glycoside-like activity in serum in conditions associated with volume expansion lends support to the view that endogenous cardiac glycoside-like substances could be important in volume homeostasis and not only in the development and maintenance of hypertension (Kelly, et al, 1986a). Kelly, et al, (1986a) measured digoxin cross-reactivity and Na^+K^+ATPase inhibition in extracts of plasma obtained from normotensives, essential hypertensives with normal renal function, hypertensives with moderate chronic renal failure (CRF) and patients with end-stage renal failure on chronic haemodialysis. The hypertensives with moderate CRF had greater levels of

Na^+K^+ATPase inhibitor and digoxin cross-reactivity than normotensives. Na^+K^+ATPase inhibitor was greater in hypertensives with moderate CRF than in hypertensives with normal renal function who in turn had higher levels than normotensives but patients on dialysis had levels similar to those of the normotensives, although dialysis acutely raised plasma Na^+K^+ATPase inhibitor levels. The substances responsible for the increased Na^+K^+ATPase inhibition were identified as NEFA's which had been designated EI_1 and EI_3 in previous work (Kelly, et al, 1985). In agreement with the results from the comparison of normotensive and hypertensive subjects reported in this thesis, Kelly, et al, found no correlation between blood pressure and Na^+K^+ATPase inhibitor or digoxin cross-reactivity in these groups of subjects.

A similar study by Deray, et al, (1986) also reported that hypertensives had higher levels of Na^+K^+ATPase inhibitor, as measured by tritiated ouabain binding, than normotensives but that normotensives with a family history of hypertension had a higher level than normotensives without such a family history. Again, no correlation was observed between Na^+K^+ATPase inhibition and blood pressure nor did inhibition correlate with plasma renin activity, however, a slight but significant correlation was obtained between inhibition and urinary sodium excretion, again supporting an association of DLIR with volume homeostasis.

Deray, et al, found that patients with chronic renal failure, who did not require dialysis, had Na^+K^+ATPase inhibitor levels which were similar to those of the normotensive control group but Kelly, et al, reported raised levels in such subjects. It was likely that the difference between these results was due to the fact that the patients studied by Deray, et al, were normotensive but those of Kelly, et al, were hypertensive which would suggest that the presence of the Na^+K^+ATPase inhibitor was associated with their hypertension. As discussed above, these two studies disagree about the effect of dialysis on Na^+K^+ATPase inhibitor levels. Kelly, et al, (1986a) report that dialysis acutely raises Na^+K^+ATPase inhibitor levels whereas Deray, et al, (1986) report a decrease after dialysis. One reason why these authors have reached different conclusions may be because they are treating their samples differently. Kelly, et al, are performing a certain amount of extraction prior to assay but Deray, et al, are using a 1/20 dilution of untreated plasma.

(b) Endogenous cardiac glycoside-like substances in hypertension

According to the natriuretic hormone hypothesis (de Wardener & MacGregor, 1982), natriuretic hormone should be present in greater amounts in essential hypertensives as compared with normotensives. There have been several

reports which show that hypertensives have a raised level of Na^+K^+ATPase inhibitor in their plasma (Hamlyn, et al, 1982; MacGregor, et al, 1981; Poston, et al, 1981; Sagnella, et al, 1986).

In the studies contained in this thesis, the inhibitory capacity of extracts from plasma samples from normotensives and hypertensives was assessed initially using a colorimetric Na^+K^+ATPase assay. A pilot study comprising plasma samples from 12 normotensives and 12 hypertensives demonstrated that it was possible to extract substance(s) which would inhibit Na^+K^+ATPase using Sep-Pak C_{18} cartridges. However, in the larger study (32 normotensives, 46 hypertensives) no measurable inhibition was extracted from the plasma. One reason for the different result obtained in the second study may have been a difference in the biochemical parameters of the two groups of hypertensives. Unfortunately, as discussed later, no such data were available. The main reason for the different results was probably methodological. The difference in the concentration factor between the two studies may have contributed to the different results obtained; in the pilot study the eluate was reconstituted in 350µl whereas in the larger study it was reconstituted in 500µl to allow sufficient eluate for measurement of digoxin cross-reactivity as well as Na^+K^+ATPase inhibition. The results from this study were not evaluated until after

all the samples had been measured so no action was taken to improve the concentration factor, although it would not have been possible to take remedial action because the way the work had been organised all samples had been extracted, pipetted into assay tubes and frozen before the first batch of samples was assayed for inhibitory activity. In subsequent work, using the enzyme-linked assay, raw data was examined immediately so this situation would never be repeated. Although there was no significant difference between the eluates and the NAF in the study, some eluates did show very small amounts of inhibition. However, these values were so small that they were encompassed within the errors in the assay. As the eluates were reconstituted in NAF, and NAF has been noted to interfere in the Na^+K^+ATPase assay, it is possible that the NAF masked any small amount of inhibition which was present. A very small amount of inhibition could also have been obscured by variation the recovery from Sep-Pak cartridges.

Although the eluates in the large study did not contain any measurable inhibitory activity they did cross-react with digoxin antibodies. This digoxin cross-reactivity did not correlate with mean arterial pressure and there was no significant difference between the results obtained for normotensives and hypertensives.

When the larger study was repeated, using the enzyme-linked

Na^+K^+ATPase assay, measurable inhibition was present in the eluates. There were two possible reasons for the different results with the two assays. First, it was possible to reconstitute the eluate in a considerably smaller volume; 170µl compared with 500µl when the colorimetric Na^+K^+ATPase assay was used, thus increasing the concentration factor achieved and so improving the resolving power. Secondly, the eluates were reconstituted in buffer, instead of NAF, because it had been observed that NAF contained substances which were capable of interfering in the Na^+K^+ATPase assay and masking all or part of the inhibition. Not only were all eluates capable of inhibiting Na^+K^+ATPase but they also showed cross-reactivity with digoxin antibodies. However, there was no correlation between the two activities supporting the idea that more than one substance was extracted. Furthermore, there was no correlation between mean arterial pressure and inhibition or cross-reactivity when expressed as ouabain or digoxin equivalents respectively.

Separation on HPLC, discussed later, provides a possible explanation for the lack of correlation between the inhibitory activity and cross-reactivity measured in the samples from normotensive and hypertensive patients. On HPLC the inhibitory activity and cross-reactivity separated into several fractions but fractions possessing both activities eluted in only two positions. It is

possible that the total concentrations of inhibitory activity and cross-reactivity could alter independently of one another so further refinement of the Sep-Pak separation method would be necessary to determine whether the fractions which show both inhibition and cross-reactivity had altered in concentration.

In their study of plasma samples from normotensives and hypertensives, Hamlyn, et al, (1982) also found no correlation between inhibition and cross-reactivity although they did find a correlation between the inhibition and blood pressure. The enzyme-linked Na^+K^+ATPase assay used in this thesis was based on the method used by Hamlyn, et al, but plasma sample treatment was different; Hamlyn, et al, deproteinised plasma by acidification and heating, whilst the Sep-Pak extraction method concentrated substances which had similar polarities, so the extracts tested in the assay by Hamlyn, et al, (1982) were likely to contain different substances from those in the Sep-Pak extract.

Blood pressure has also been correlated with levels of digoxin cross-reactivity in monkeys (Gruber, et al, 1982) and with the ability to inhibit ouabain binding to erythrocytes in hypertensive patients (Sagnella, et al, 1986). MacGregor, et al, (1981) reported that serum from hypertensives contained more inhibitory activity, as

assessed by the capacity to stimulate glucose-6-phosphate dehydrogenase, than serum from normotensives. However, my findings are in agreement with those of Kelly, et al, (1986a) and Deray, et al, (1986) who found no correlation between ouabain-like activity and mean arterial pressure.

The different conclusions, regarding the relationship between cardiac glycoside-like activity and blood pressure, are a consequence of the lack of concensus about sample treatment and measurement of cardiac glycoside-activity and the problems associated with studying the effect of serum or plasma on the assays. Serum and plasma contain many substances which, if concentrated sufficiently, will interfere in assays for cardiac glycoside-like activity. For example, Na^+K^+ATPase can be inhibited by potassium (Glynn and Karlish, 1975) or NEFA's (Bidard, et al, 1984), endogenous steroids could cross-react in immunoassays. In the literature discussed above, workers using similar assays but different sample preparation arrived at different conclusions. Both Deray, et al, (1986) and Sagnella, et al, (1986) were measuring ouabain binding to red blood cells by similar methods but whereas Deray, et al, boiled the plasma, then assayed it at a 1/20 dilution and found no correlation with blood pressure, Sagnella, et al, used Dowex resin to remove potassium ions, a possible source of inhibition, and noted a correlation of ouabain-like activity with systolic blood pressure. Another

possible source of difference was subject selection. Deray, et al, suggested that their results differed from those of Hamlyn, et al, (1982) because the sex and ethnic background of the two groups of subjects were different.

It has been shown by several workers (Devynck, et al, 1983; Cloix, et al, 1983; Deray, et al, 1986; Kelly, et al, 1986a) that there are not simply two separate populations; normotensives and hypertensives, but that there is a spectrum of values which can be divided up into hypertensives, normotensives with a family history of hypertension and normotensives. This should not necessarily be surprising since blood pressure follows a normal distribution within a given population. Therefore, if this factor contributes to the raised blood pressure of some of the population then it should not be expected to be either simply present or absent. The results contained in this thesis would support this view since no significant difference was found between normotensives and hypertensives. Insufficient information was available to divide the normotensives into those with a family history of hypertension and those without.

As discussed earlier, it has been reported that raised levels of cardiac glycoside-like activity are present in conditions associated with volume expansion. For this reason it would have been valuable to have information

about the renal and liver function of the subjects. The renin status of the subjects would also have been of interest. If some of the subjects had low-renin hypertension the natriuretic hormone would be expected to be present at higher concentrations than in subjects whose renin levels were normal. However, Deray, et al, (1986) did not find a correlation between inhibition of [^{3}H]-ouabain binding and renin activity.

Animal models of hypertension have been investigated for the presence of ouabain-like substances but these models, like human essential hypertension, have different characteristics (Genetic rat models for hypertension, 1987); some are volume expanded, others are not, some are spontaneously hypertensive, others require a high salt diet to initiate the development of hypertension. However, cardiac glycoside-like activity has been measured in a number of different rat models. Lichtstein, et al, (1985a) found no difference between the amount of inhibitor found in HPLC purified extracts from the serum of Sabra hypertension prone and hypertension resistant rats. In the DOCA-salt rat, ouabain-like activity, as measured by [^{3}H]-ouabain displacement from brain synaptosomes, was greatest in DOCA-salt rats, less in salt treated rats and least in untreated rats (Kojima, 1984). Digoxin cross-reactivity was observed in DOCA-salt rats only. This activity was related to the blood pressure since the blood

pressure was significantly higher in DOCA-rats than in control and salt treated rats. It has also been shown that injection of digoxin antibodies into DOCA-salt hypertensive rats results in a reduction in their blood pressure (Kojima, et al,1982).

In recent work with the Milan hypertensive strain, it was shown that raised levels of an endogenous Na^+K^+ATPase inhibitor were present in the plasma of young genetically hypertensive rats even before there was any increase in their blood pressure (Holland, et al, 1987). In this strain of rat it has been previously demonstrated that, when kidneys were transplanted from young rats before they had developed high blood pressure into rats which should remain normotensive, the hypertension "followed the kidney" and the recipient rat developed high blood pressure (Bianchi, et al, 1974). The work of Holland, et al, (1987) supports the hypothesis that a genetic kidney defect results in stimulation of the release of an endogenous Na^+K^+ATPase inhibitor which leads to the development of hypertension.

Holland, et al, were using the cytochemical assay in which inhibition of Na^+K^+ATPase was measured by its ability to stimulate glucose-6-phosphate dehydrogenase. This assay was also used by Millett, et al, (1986), who found greater quantities of glucose-6-phosphate dehydrogenase stimulating

activity in the plasma and acetone extract of hypothalami from spontaneously hypertensive rats as compared with normotensive rats. Millett, et al suggest that the hypothalamus could be a source of the inhibitory substances found in the plasma. However, it was reported recently (Doris, 1988) that plasma levels of a digoxin-like substance decreased following adrenalectomy in the rat which suggests that the adrenal gland could also contribute to the plasma concentration of digoxin-like substances. This work supports the results reported in this thesis and contrasts with the report that the adrenal gland was devoid of inhibitory activity as assessed, indirectly, by the ability to stimulate glucose-6-phosphate dehydrogenase (Alaghband-Zadeh, et al, 1983).

3. CONCENTRATION AND SEPARATION OF A FACTOR FROM SERUM

The digoxin cross-reactivity and inhibitory activity extracted from plasma, using the Sep-Pak extraction technique, was concentrated and then separated into several active fractions by HPLC.

(a) Concentrated Eluates

To investigate the dose-response of the inhibition and cross-reactivity, the Sep-Pak eluates were further concentrated by application to a second Sep-Pak cartridge.

These concentrated eluates were prepared from a considerably larger volume of plasma than the single patient samples used for the comparison of normotensives and hypertensives. When pooled normotensive plasma was concentrated 120-fold the beginning of a dose-response curve was obtained for inhibition. Polycythaemic plasma was concentrated to a greater extent (344-fold) and did not show any dose-response for inhibition but a curve was obtained for cross-reactivity with digoxin antibodies. This was the first indication that the inhibition and cross-reactivity could be dissociated from one another and is consistent with the finding, reported earlier (p158) that there was no correlation between the inhibitory activity and cross-reactivity extracted from normotensive and hypertensive subjects. It should be noted that since the eluates in these studies were reconstituted in NAF some of the inhibition may have been masked.

It was difficult to obtain large volumes of human plasma so bovine serum was investigated for the presence of ouabain-like substances since it was available in large volumes from a local abattoir and it has been reported that the plasma from a volume expanded cow contained natriuretic activity (Lichardus, et al, 1968). A large volume (approx. 3.4l) of bovine serum was concentrated on Sep-Pak cartridges to give a final concentration factor of 400. Dilutions of this produced a dose-response curve in both

the Na^+K^+ATPase assays and in the digoxin RIA.

When the values obtained using the colorimetric and the enzyme-linked Na^+K^+ATPase assays were compared the % inhibition for a given concentration factor were identical. Since the ouabain standards used to quantify the inhibition in the two assays cover different ranges, the inhibitory activity would be assigned different ouabain equivalents depending on the assay used.

The digoxin cross-reactivity of the bovine serum concentrated eluate was assayed using several different commercial kits and the "in-house" method. The eluate produced measurable cross-reactivity in all kits but to a different extent depending on the assay. The difference in cross-reactivity reflects the different affinities of the antibodies used in each method.

(b) HPLC of bovine serum eluate

When the eluate prepared from bovine serum was separated using HPLC on an octadecylsilane column, fractions capable of Na^+K^+ATPase inhibition eluted as three peaks and fractions which cross-reacted with digoxin antibodies eluted as three peaks. The largest peak of Na^+K^+ATPase inhibition did not show any cross-reactivity but the other two peaks, which eluted at the beginning of the gradient,

had both inhibitory activity and cross-reactivity. The levels of inhibition were very low and to obtain peaks with a higher activity it would be necessary to repeat this using an eluate which was more concentrated. As there was insufficient sample to repeat the separation, it was not possible to make any comment regarding the reproducibility of this pattern of peaks. However, HPLC separation of adrenal cortex cytosol eluate, which will be discussed later and which used the same equipment, protocol, solvents and acetonitrile gradient was very reproducible (10 runs). The HPLC profile obtained for the concentrated serum could be used as a guide to indicate the acetonitrile concentrations suitable for the elution of the two peaks showing both inhibitory capacity and cross-reactivity from Sep-Pak cartridges. Having extracted and concentrated the substances in this way they could then be applied, separately, to the HPLC column and eluted using a suitable gradient. If this proved successful with bovine serum the Sep-Pak method could then be applied to samples from hypertensive and normotensive subjects.

Tal, et al, (1986) have also extracted a substance from bovine plasma which was capable of inhibiting Na^+K^+ATPase. After exhaustive solvent extraction and HPLC on an amino column a single peak was obtained. They were careful in their protocol to keep the plasma at temperatures less than 40^oC throughout the extraction and suggest that the

multiple peaks reported by other workers could be artefacts caused by the raised temperature during sample preparation. My results do not lend support to this idea, since the samples were never boiled and the methanol was evaporated off the eluate at a temperature of 37°C, but should be considered in the interpretation of other studies in which plasma was heated and the ouabain-like activity was subsequently separated into several active fractions. Artefacts could also arise because substances normally present in plasma have been concentrated to abnormally high levels e.g. potassium, NEFA's.

Plasma collected from normotensive subjects was boiled and separated on HPLC by Kelly, et al, (1985). This resulted in the isolation of 4 different peaks; labelled EI_1, EI_2, EI_3, IR_1, which were later identified as NEFA, in the case of EI_1 and EI_3, and half NEFA and half lysophospholipid for EI_2. IR_1 showed only cross-reactivity and was thought to be due to some NEFA but mostly due to hydrocortisone or other endogenous steroids. Crabos, et al, (1984) also report that plasma could be separated into several active peaks of activity using HPLC, as could urine. Ouabain-like activity in plasma has also been separated into several peaks using anion exchange chromatography (Cloix, et al, 1984). These results form further support for the idea that more than one substance is responsible for the activity present in plasma. When pooled plasma from DOCA-

salt hypertensive rats was separated by gel filtration on Sephadex G-25, two peaks were obtained; one peak showed inhibition and cross-reactivity whilst the other would only cross-react, suggesting that at least two substances were responsible for the cross-reactivity present in the plasma of DOCA-salt rats (Kojima 1984). The antibody used by Kojima was reported to show no cross-reactivity with adrenal steroids (Kojima, 1984) so it is unlikely that the peak which exhibited only cross-reactivity was due to DOCA.

As it is proposed that natriuretic hormone is released in response to volume expansion (de Wardener, 1982), several groups have studied animals which have been experimentally volume expanded. Gruber, et al, (1980) found more inhibitory activity and digoxin cross-reactivity in the serum of volume expanded dogs than in hydropenic dogs. Tamura, et al, (1985) identified the substances responsible for some of the inhibitory activity in the serum of volume expanded hogs as linoleic and oleic acid. These NEFA were reported to be present in the HPLC fractions isolated by Kelly, et al, (1986b). Using column chromatography, reverse phase and gel filtration HPLC, Goto, et al, (1988) isolated a factor from volume expanded dog plasma which had similar polarity to ouabain and had a molecular weight, according to fast atom bombardment mass spectrometry (FAB mass spec.), of around 343Da. The elution pattern of NEFA on reverse phase HPLC was different to that of the factor

they isolated and NEFA were not found to have any effect on their assay systems so it was considered unlikely that their isolated substance was a NEFA.

Prior to dialysis, renal patients are volume expanded. Dasgupta, et al, (1987) extracted two fractions, from the plasma ultrafiltrate obtained during dialysis, which showed both inhibition and cross-reactivity. Both of these fractions were analysed by FAB mass spec. and both had a dominant component at 531Da. One of these fractions was further analysed and identified as a novel compound derived from lysophosphatidyl serine with a novel 19:4 fatty acid side chain (Dasgupta, et al, 1988). However, the authors did not discount the possibilty that this compound was an artefact of the isolation procedures which comprised alkaline solvent extraction followed by HPLC.

Extraction with acetone has been shown to produce artefacts under certain conditions (Whitmer, et al, 1982). Using acetone extraction, a digoxin-like substance was isolated from guinea pig brain (Fishman, 1979) and Haupert and Sancho (1979) extracted a substance with similar properties from bovine hypothalamus. Millett, et al, (1987) reported that greater levels of inhibitory substance were present in the hypothalami of SHR compared with the hypothalami of the normal controls, in this work acetone was employed in the extraction procedure.

Haupert, et al, (1984) modified their extraction procedure and demonstrated that they had isolated a reversible inhibitor of Na^+K^+ATPase from bovine hypothalami which was shown subsequently to inhibit ^{86}Rb uptake and [^{3}H]-ouabain binding to intact, cultured renal epithelial cells (Cantiello, et al, 1988). Cultured rat hypothalamic cells have been employed to avoid the problems associated with tissue extraction. These cells were found to release a Na^+K^+ATPase inhibitor which eluted as a single peak from HPLC (Morgan, et al, 1985) thus supporting the idea that the endogenous cardiac glycoside-like substance(s) is produced in the hypothalamus. This factor was recently shown to produce a concentration-dependent increase in tension in de-endothelialised rabbit aortic strips (Mir, et al, 1988); an effect which could be reversed by washing. This factor also potentiated the vasoconstrictor effect of noradrenaline and caused greater calcium retention than control medium. If it is demonstrated that this factor is released into the circulation and has a natriuretic effect it could well be the putative hormone which has been sought.

4. BOVINE ADRENAL CORTEX

(a) HPLC of adrenal cortex cytosol eluate

Digoxin cross-reactivity has been reported in the adrenal

gland of the rat (Schreiber, et al, 1981a) and of the rabbit (Schreiber, et al, 1981b). In the rabbit, two fractions were separated by thin layer chromatography which showed both cross-reactivity and the ability to inhibit ^{86}Rb uptake into erythrocytes (Schreiber, et al, 1981b). Organ surveys in rats have found the highest levels of digoxin cross-reactivity in the adrenal gland (Castenada-Hernandez and Gotfraind, 1984; Valdes, et al, 1985). However, the adrenal gland has been reported to be devoid of the ability to inhibit Na^+K^+ATPase, as assessed by the ability to stimulate glucose-6-phosphate dehydrogenase (Alaghband-Zadeh, et al, 1983), and of all the organs surveyed by these authors only the hypothalamus contained stimulatory activity. Two more recent organ surveys, published after completion of work on this thesis, have confirmed that the adrenal gland does contain activity. Rauch and Buckalew (1988) reported that, of the peripheral organs, the adrenal gland contained the most activity, as measured as the ability to inhibit [^{3}H]-ouabain binding to red blood cells. Various regions of the brain were also examined and the striatum was reported to have similar concentrations of activity to the adrenal gland. Another recent organ survey reported that of all tissues, including the brain, only the adrenal gland showed digoxin cross-reactivity (Doris, 1988). The difference between the results obtained in these organ surveys may due to the

different extraction procedures or due to the different assays used.

To investigate whether the adrenal gland contained substances capable of both inhibitory activity and cross-reactivity, adrenal cortex cytosol was prepared and separated using the Sep-Pak extraction procedure. It was observed that Sep-Pak eluate was capable of inhibiting Na^+K^+ATPase and cross-reacting in the digoxin RIA in a dose-dependent manner. The presence of such high levels of inhibitory activity and cross-reactivity in the adrenal cortex suggested that it may be a suitable model for investigation of extraction methods intended for use on plasma samples, however the HPLC profile obtained from adrenal preparations was not sufficiently similar to that of serum for this to be the case (Fig.3.6 and Fig.3.8).

HPLC of bovine adrenal cortex cytosol resulted in several peaks of inhibition and cross-reactivity which eluted in different positions to those obtained by HPLC separation of serum. The pattern of peaks was extremely reproducible (10 runs) and in only two positions did the inhibition and cross-reactivity co-elute. This is similar to the serum results, in that two co-eluting peaks were obtained, however the activity from serum eluted in different positions. Fractions with cortisol cross-reactivity eluted in the same position as those with digoxin cross-

reactivity except for one extra cortisol peak. The largest cortisol peak was thought to be native cortisol and eluted immediately before the fractions of interest.

Endogenous digitalis-like factors purified from plasma have been identified as NEFA, phospholipid and endogenous steroids (Kelly, et al, 1986b). For this reason fractions from the HPLC of bovine adrenal cortex were assayed for NEFA using a commercial kit. NEFA eluted in several peaks before and one peak after those of interest. The last peak of NEFA co-eluted with a peak of inhibition which did not show any cross-reactivity. Since the kit which was used was capable of detecting several different NEFA's, and the values obtained for the HPLC fractions were all less than the bottom standard it would appear that the HPLC method concentrates the small quantity of NEFA extracted on the Sep-Pak cartidges into discrete peaks thus separating them from the substances of interest.

Cloix, et al, (1985) have identified a substance with digoxin-like characteristics which appears to be an amino-glyco-steroid. The HPLC peak fractions could be steroid-like since they cross-react in both the cortisol and digoxin RIA's and so could be structurally similar to these molecules.

The two peaks from HPLC of bovine adrenal cortex were

subjected to tryptic digestion and heat (80°C, 10min). Heat had no effect on either of the peaks but trypsin approximately halved the activity of both peaks. This would suggest that the peaks contain a heat stable peptide. Unfortunately there was insufficient sample to measure the digoxin cross-reactivity after tryptic digestion which would have helped to resolve the question of whether there was more than one substance responsible for the cross-reactivity and the inhibition caused by the peaks. It is possible that the inhibition may be due to several substances in both peaks, only one or some of which is susceptible to trypsin, however, all would appear to be capable of withstanding 80°C for 10 min.

In support of the findings of this thesis is the report of Tamura, et al, (1987) who purified a single peak of ouabain-like activity from whole bovine adrenal glands by homogenisation in methanol followed by HPLC using several different columns and solvent conditions. This factor inhibited Na^+K^+ATPase, displaced [^{3}H]-ouabain and inhibited [^{86}Rb]-uptake into red blood cells. Dose-response curves for these activities were parallel to those of ouabain and the inhibition of Na^+K^+ATPase by the factor was shown to have the same dependence on potassium concentration as ouabain. Although the data was not given, it also apparently cross-reacted with digoxin antibodies and the activity was unaffected by incubation with pronase E,

trypsin, deoxyribonuclease I, ribonuclease A, phospholipase C, weak alkali or heat treatment but was destroyed by hydrochloric acid (6M, 115^{o}C, 21h). FAB mass spec. indicated a molecular mass of 336Da.

It is interesting that Tamura, et al, found only a single peak whereas in my work the inhibition and cross-reactivity co-eluted in two peaks. It could be that one of the peaks would not displace ouabain from $Na^{+}K^{+}$ATPase or inhibit ^{86}Rb uptake from red blood cells. Clearly this would merit further investigation. Since the peak fractions have been subjected only to one HPLC separation it is likely that there is more that one substance present in each peak, an assumption borne out by the results from the tryptic digestion, it might be that one of the peak fractions contains the factor isolated by Tamura, et al. Resistance to tryptic digestion was also a characteristic of the hypothalamic factor recently shown to increase the tension of de-endothelialised aortic strips (Mir, et al, 1988) and it is possible that the factor in the adrenal gland and in the hypothalamus are related, although Tamura, et al, do not consider their adrenal factor to be a peptide whereas the hypothalamic factor of Mir, et al, is thought to be a peptide.

(b) Effect of adrenal peak fractions on steroidogenesis in isolated bovine adrenal cortex cells

The HPLC peak fractions produced as much as 50-fold stimulation of steroid production in isolated adrenal cells. The first peak stimulated aldosterone production only, but the second peak stimulated cortisol, corticosterone and aldosterone production. The effects that the HPLC peaks had were in contrast to the effect of ouabain on steroidogenesis. Ouabain has been reported to inhibit aldosterone synthesis in bovine zona glomerulosa cells (Elliot et al, 1986) and in dog adrenal cortex slices (Cushman, 1969) but either stimulated or inhibited steroidogenesis in rat zona glomerulosa cells depending on the concentration used (Schriffrin, et al, 1981; Brayley and Williams, 1978). In rat zona glomerulosa cells, 10^{-5}M ouabain stimulated basal aldosterone production and corticosterone production but concentrations greater or less than this had no effect on basal steroid production (Brayley and Williams, 1978). 10^{-5}M ouabain increased aldosterone production in AII-stimulated rat zona glomerulosa cells above the already raised levels, but concentrations greater than this inhibited the response to AII, ACTH and potassium (Brayley and Williams, 1978; Schriffrin, et al, 1981). The adrenal cell work reported in this thesis was performed on bovine material and so according to the results of Elliot, et al, (1986) a

substance with ouabain-like qualities might be expected to inhibit steroidogenesis. Since the opposite was found to be the case a possible explanation could be that the ouabain-like substances present in the HPLC peak fractions act on bovine adrenal cells in manner analagous to that of ouabain on rat adrenal cells and that, at the concentration tested, they stimulate steroidogenesis but at a different concentration they would inhibit steroidogenesis.

The stimulation obtained with these substances was far greater than the stimulation due to AII or ACTH, which may indicate that the peaks contain steroid precursors of those which were measured. To elucidate whether the substances present in the HPLC fractions act as agonists, cAMP and cGMP could be measured following incubation of adrenal cells with the fractions. The concentration of the second messenger would be expected to increase if the cardiac glycoside-like substances are acting as agonists through interaction with receptors on the cell. If the substances in the HPLC fractions are steroid precursors then the concentration of second messenger would not be expected to increase.

Another complication was the cross-reactivity of the HPLC fractions with the antibodies for cortisol and corticosterone. The cross-reactivity was so great that some of the supernatants from the cell incubations had to

be assayed at dilutions as high as 1/1000 in the corticosterone assay and 1/100 in the cortisol assay. Cross-reactivity with the aldosterone assay was minimal and presented no problem. There was insufficient sample to perform dilution curves in the cortisol and corticosterone assays to check that the cross-reactivity diluted out in parallel with the native steroid. This meant that the absolute values for the stimulation would not be as great as they appear if the cross-reactivity did not dilute out in parallel.

Further study of the activity in the adrenal gland could follow several lines. The Sep-Pak extraction method could be modified by using the acetonitrile concentrations at which the peak fractions elute from the HPLC column in place of methanol. This would provide a better way of concentrating the substances of interest prior to HPLC separation. HPLC conditions could also be altered by using either the same type of column with a different gradient system or on a different type of column which would separate substances according to, for example, charge. By comparing the elution position from the HPLC column of tritiated steroids with that of the peak fractions it would be possible to conclude whether or not native steroids were responsible for the activities measured. If the HPLC fractions contain cardiac glycoside-like substances then they should be capable of

inhibiting the binding of tritiated ouabain to either purified Na^+K^+ATPase or red blood cells therefore future work should include the development of a radioreceptor assay.

5. CONCLUSION

The major problem in attempting to isolate cardiac glycoside-like substances has been that it was necessary to concentrate the sample several hundred fold thus making it more likely that artefacts could be produced. Artefacts could arise because substances normally present in tissue or plasma have been sufficiently concentrated to inhibit the enzyme, for example, potassium (Glynn and Karlish, 1975) or ascorbic acid (Ng, et al, 1985). Some compounds which have been shown to possess ouabain-like qualities include steroid compounds such as chlormadinone acetate (Hnatowich and LaBella, 1984), dehydroepiandrosterone sulphate (Vasdev, et al, 1985), methylguanidine (Minkoff, et al, 1972), non-esterified fatty acids (Kelly, et al, 1986b; Bidard, et al, 1984); Tamura, et al, 1985) and a novel cytochrome P_{450} metabolite (Schwartzman, et al, 1985). By using cultured hypothalamic cells (Morgan, et al, 1985) the problems of producing artefacts are reduced.

However, there is now a considerable body of evidence which supports the existence of endogenous Na^+K^+ATPase inhibitors

and the involvement of these substances in hypertension and certain conditions where there is a disorder of fluid balance. Since it is likely that essential hypertension has several different causes it is plausible that the natriuretic hormone hypothesis provides an explanation of one of the ways in which hypertension may develop and be maintained. Much evidence supports the idea that the endogenous Na^+K^+ATPase inhibitors are produced by the hypothalamus and several groups have isolated Na^+K^+ATPase inhibitor from this organ (Haupert, et al, 1984; Millett, et al, 1986; Morgan et al, 1985;). However, cardiac glycoside-like substances have also been isolated from the adrenal gland (Tamura, et al, 1987), the heart (Fagoo, et al,1986), as well as urine (Cloix, et al, 1985), plasma (Tal, et al,1986) and amniotic fluid (Graves and Williams, 1984). The origin of the substances isolated from plasma has yet to be elucidated but the brain and the adrenal glands are prime candidates. It has been demonstrated that the natriuretic response to volume expansion was lost following decapitation but not after adrenalectomy (Kaloyanides, et al, 1977) but it has also been reported that plasma levels of digoxin cross-reactivity decrease following adrenalectomy in rats (Doris, 1988). These two reports could be reconciled if the different activities measured by different researchers were the properties of a family of cardiac glycoside-like substances which possess all or some of the properties which have been reported;

natriuretic ability, inhibition of Na^+K^+ATPase, inhibition of [^{3}H]-ouabain binding, cross-reactivity with antibodies to digoxin or ouabain. It is also possible that the substance released from the adrenal could be under the control of the hypothalamus.

SUMMARY OF WORK AND CONCLUSIONS

1. Plasma from normotensives and hypertensives was found to contain methanol-soluble substances which both inhibited Na^+K^+ATPase and cross-reacted with digoxin antibodies. There was no difference in activities between the two populations.
2. There was no correlation between the inhibition and the cross-reactivity. This conclusion is supported by some studies performed concurrently with this thesis.
3. At present, there is no concensus with regard to the correlation between the activities. The reasons for this include methodological differences which have already been discussed.
4. The activity present in serum was separated on HPLC into several fractions some of which showed both inhibitory activity and cross-reactivity. Others possessed only one activity. This is consistent with the majority opinion in the literature.
5. The adrenal gland was found to contain considerable activity which was also separated into several active

fractions by HPLC. As with separation of serum, some fractions had both inhibitory activity and cross-reactivity, others had only one activity.

6. The adrenal fractions which showed both inhibitory activity and cross-reactivity did not contain NEFA but the activity may be due to a steroid-like substance since the fractions were found to cross-react with digoxin, cortisol and corticosterone antibodies. The inhibitory capacity of the fractions was stable to heat (80^{o}C, 10min) but approximately half of the activity was lost following incubation with trypsin.

7. The active fractions from the adrenal produced a marked stimulation of steroid production. The results should be interpreted with caution as the fractions may contain steroid precursors. Further work is required on the nature of these fractions.

APPENDIX I

REAGENTS

The following is a list of chemicals, enzymes, solvents and radio-labelled compounds and their suppliers. All reagents were of analytical grade.

Alpha Laboratories, 40 Parham Drive, Eastleigh, Hants., UK

Wako NEFA C test kit

Amersham International plc, White Lion Road, Amersham, Bucks., UK

[^{125}I]-cortisol

BDH Ltd., Burnfield Avenue, Thornliebank, Glasgow, UK.

Ammonium acetate, citric acid monohydrate, disodium hydrogen phosphate, gelatin, glacial acetic acid, glucose, hydrochloric acid (conc.), magnesium sulphate, methanol, potassium dihydrogen phosphate, sodium azide, sodium chloride, sodium hydroxide, 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)ethanesulphonic acid (TES).

CMD(UK) Ltd., 12 Yeomans Park, Yeomans Way, Bournemouth, UK

[^{125}I]-digoxin

Ciba Corning Diagnostics Ltd., Halstead, Essex, UK

[^{125}I]-digoxin

Flow Laboratories, Woodcock Hill, Harefield Road, Rickmansworth, Herts., UK

Medium 199 (modified) with Earles's salts, with glutamine, without bicarbonate.

ICN Biomedical Ltd., Free Press House, Castle Street, High Wycombe, Bucks., UK

Pentex^{R} bovine serum albumin, fraction V

Immunodiagnostics Ltd., Usworth Hall, Washington, Tyne & Wear, UK

[^{125}I]-aldosterone and [^{125}I]-corticosterone

Lorne Diagnostics Ltd., Unit 11, Cratfield Road, Moreton Hall Industrial Estate, Bury St.Edmunds, Suffolk, UK

Collagenase (type I)

NBL Ltd., Unit 11, South Nelson Industrial Estate, Cramlington, Northumberland, UK

Earle's balanced salt solution

Rathburn Chemicals Ltd., Walkerburn, Scotland, UK

HPLC grade solvents; acetonitrile (S grade), methanol, water.

Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Scotland, UK

Sheep anti-digoxin antiserum, sheep anti-cortisol antiserum, normal sheep serum, donkey anti-sheep IgG precipitating serum.

Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, UK.

Adenosine 5'-triphosphatase (dog kidney), adenosine 5'-triphosphate (disodium salt, vanadium free), aldosterone, ascorbic acid (free acid), ammonium molybdate tetrahydrate, bovine serum albumin, charcoal (activated), ethylene glycol-bis(β-aminoethylether)N,N,N',N',- tetraacetic acid (EGTA), imidazole, L-lactic dehydrogenase, magnesium chloride, β-nicotinamide adenine dinucleotide (NADH, reduced form), ouabain octahydrate, pepstatin A, phenylmethylsulphonyl fluoride (PMSF), phosphoenolpyruvate (tricyclohexylammonium salt), potassium chloride, potassium phosphate, pyruvate kinase, sodium arsenite, sodium chloride, trisodium citrate, trichloroacetic acid, tris(hydroxymethyl)aminomethane (Tris), N-tris(hydroxymethyl)methyl 2-aminomethane sulphonic acid (TES), trypsin, trypsin inhibitor (soya bean).

APPENDIX II

COMPOSITION OF BUFFERS AND REAGENTS

All buffers and reagents were prepared using distilled water and stored at 4°C.

REAGENTS FOR COLORIMETRIC Na^+K^+ATPase ASSAY

Imidazole buffer, 10mM, pH 7.4

Imidazole (10mM), sodium chloride (100mM), potassium chloride (5mM), magnesium chloride (12mM), EGTA (5mM) - pH to 7.8 with conc. sodium hydroxide.

Arsenite-citrate reagent

To 10g trisodium citrate and 10g of sodium arsenite dissolved in 400ml distilled water, add 10ml glacial acetic acid and make up to final volume of 500ml.

REAGENTS FOR ENZYME-LINKED Na^+K^+ATPase ASSAY

The Cobas FARA adds diluent (distilled water) to the cuvettes in addition to sample and reagent so it is necessary to allow for this dilution when making up reagents.

Tris-TES-salts buffer (TTS), 40mM, pH 7.4 (37°C)

Salt	Concentration in buffer/mM	Concentration in cuvette/mM
Tris	53	40
TES	53	40
EGTA	6.6	5
sodium chloride	132	100
potassium chloride	26	20
magnesium sulphate	5.9	4.5

Add EGTA to distilled water (approximately one tenth to one fifth of the final volume) pH to approximately 7 with concentrated sodium hydroxide. When dissolved add this to a solution containing the other salts. This should have a pH of 7.8 at room temperature (20°C) which is equivalent to a pH of 7.4 at 37°C.

FARA Assay cocktail

Reagent	Concentration in cocktail/mM	Concentration in cuvette/mM
ATP (disodium salt)	5	3
PEP (tricycohexyl-ammonium salt)	2	1.2
NADH	0.42	0.25
Lactate dehyrogenase	17 U/ml	10 U/ml
Pyruvate kinase	17 U/ml	10 U/ml

TTS and assay cocktail (minus enzymes and NADH) can be prepared and stored at 4°C for one month. Solutions containing NADH and enzymes must be prepared and used on the same day, these cannot be stored.

OTHER BUFFERS

Ammonium acetate, 20mM, pH 7.4

Make up a 20mM solution of ammonium acetate and pH to 7.4 using ammonia solution.

Homogenising buffer, 50mM, pH 7.4 (4°C)

Combine Tris (50mM), EGTA (1mM), PMSF (0.2mM), pepstatin A (0.05pg/l) and pH to 7 with conc.HCl. First dissolve PMSF in a small volume (approx. 3-5ml/l buffer) of ethanol and add to a solution containing Tris and EGTA. Add pepstatin A after the buffer has been brought to pH 7 (equivalent to pH 7.4 at 4°C).

Tris-buffered saline, 50mM, pH 7.4 (37°C)

Tris (50mM) and sodium chloride (140mM), pH to 7.8 (20°C) with conc.HCl. This will have a pH of 7.4 at 37°C.

DIGOXIN RIA REAGENTS

Phosphate buffer, 50mM, pH 7.2

Prepare 50mM phosphate buffer by adding 50mM potassium dihydrogen phosphate to 50mM disodium hydrogen phosphate to give a pH of 7.2. To 1l of this add 5.0g BSA, 1.0g sodium azide and 58g sodium chloride.

Pre-precipitated antibody reagent

In a universal container combine 11ml phosphate buffer, 50µl sheep anti-digoxin antiserum (SAPU), 940µl normal sheep serum (SAPU), 12ml donkey anti-sheep IgG (SAPU). Mix well and leave overnight at 4°C. Centrifuge (200 x g, 5min) and decant supernatant. Resuspend precipitate in

20ml phosphate buffer and centrifuge (200 x g, 5min). Decant supernatant and resuspend precipitate in 20ml phosphate buffer. For use in digoxin RIA, add contents of universal to 160ml phosphate buffer.

CORTISOL RIA REAGENTS

Citrate buffer, 0.1M, pH 4

For 0.1M citrate buffer, pH 4, dissolve 24.8g citric acid (monohydrate) and 24.1g trisodium citrate (dihydrate) in distilled water and make up to 2l. Dissolve 4g gelatin in approx. 200ml buffer with gentle heat and stirring. Allow to cool, add back to the rest of the buffer (final gelatin concentration = 0.2%) and add 0.13g sodium azide.

Pre-precipitated antibody reagent

In a universal container combine 650µl sheep anti-cortisol antiserum (SAPU), 530µl normal sheep serum (SAPU), 15ml donkey anti-sheep IgG (SAPU), 10ml citrate buffer. Mix well and store overnight at 4°C. Centrifuge (200 x g, 5min) and decant supernatant. Resuspend precipitate in 20ml citrate buffer and centrifuge (200 x g, 5min). Decant supernatant and resuspend precipitate in 20ml citrate buffer. For use in cortisol RIA, dilute to final volume of 500ml.

REAGENTS FOR CORTICOSTERONE RIA AND ALDOSTERONE RIA

Phosphate buffer, 50mM, pH7.4

Prepare 50mM phosphate buffer, pH7.4, from disodium hydrogen

phosphate dodecahydrate (14.5g/l) and potassium dihydrogen phosphate (1.29g/l).

Charcoal suspension

Dissolve gelatin (0.06%) in phosphate buffer using gentle heat and stirring. Once dissolved, cool and add dextran (0.06%) and charcoal (0.6%).

APPENDIX III

PREPARATION OF CYTOSOL FROM BOVINE ADRENAL CORTEX

Adrenal glands were transported to the laboratory on ice and kept on ice between dissection and homogenisation and while waiting to be centrifuged.

Procedure

Remove fat from individual glands

↓

Cut gland in half and scrape away medulla

↓

Roughly chop glands and homogenise in 3 volumes of homogenising buffer (Appendix II)

↓

Centrifuge homogenate (15 000 x g, 1h, 4°C)

↓

Filter supernatant through gauze and centrifuge (100 000 x g, 45min, 4°C)

↓

Filter supernatant through glass wool and store overnight at 4°C.

APPENDIX IV

ABBREVIATIONS

The following is a list of the abbreviations used in this thesis.

A_{340}	absorbance value at 340nm
A_{700}	absorbance value at 700nm
AII	angiotensin II
ACTH	adrenocorticotrophic hormone
ASF	aldosterone stimulating factor
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
AV3V	anteroventral third ventricle
BSA	bovine serum albumin
C_{18}	octadecylsilane
CRF	chronic renal failure
CSF	cerebrospinal fluid
CV	coefficient of variation
DLIR	digoxin-like immunoreactivity
DOCA	deoxycorticosterone acetate
EBS	Earle's balanced salt solution
EHT	essential hypertension
FAB mass spec.	fast atom bombardment mass spectrometry
HPLC	high performance liquid chromatography
IHA	idiopathic hyperaldosteronism
M199	medium 199
MAP	mean arterial blood pressure

MRC	Medical Research Council
Na^+K^+ATPase	sodium potassium ATPase
NADH	β-nicotinamide adenine dinucleotide (reduced)
NAD^+	β-nicotinamide adenine dinucleotide (oxidised)
NAF	non-adsorbed filtrate
NEFA	non-esterified fatty acids
RIA	radioimmunoassay
SHR	spontaneously hypertensive rats
TBS	tris-buffered saline
TLC	thin layer chromatography
TTS	tris-TES-salts buffer

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PUBLICATIONS

Improvements in precision and detection limit in a sensitive phosphate microassay

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Abstract

A sensitive microassay for phosphate showed imprecision both on a phosphate standard curve and on a ouabain inhibition curve. This imprecision could be due to formation of a phosphate-independent complex when arsenite-citrate reagent addition is delayed, or to incomplete formation of the desired phosphomolybdate complex, caused by inadequate mixing. These possibilities were investigated by delayed addition of arsenite-citrate reagent and by mixing reagents after the final addition (terminal mixing) or mixing after each reagent was added (sequential mixing).

It was concluded that sequential mixing both improves precision and decreases effective detection limit.

Introduction

Micro-colorimetric assays for inorganic phosphate, dependent on the formation of a phosphomolybdate complex, have been available since the early 1950s. Measurement of inorganic phosphate by this technique involves precipitation of the phosphomolybdate with a basic dye at a low pH, then redissolving it in acetone and measuring the absorbance. Alternatively, the absorbance of the phosphomolybdate complex can be measured after treatment with a reducing agent.

The first basic dye to be used in a micro-assay for phosphate was safranin [1]. By replacing safranin with malachite green, and using detergent to prevent the dye-phosphomolybdate complex from precipitating out, Itaya and Ui [2] were able to determine inorganic phosphate concentrations as low as 1 nmol.ml^{-1}. Further improvements on this assay [3] made it possible to measure inorganic phosphate in the 0.2-16 nmol.ml^{-1} range.

Increasing absorbance of samples and blanks with time, was noted by Lanzetta *et al* [4]. This problem was overcome, while retaining sensitivity, by modifying a quenching procedure developed for use in an assay where phosphomolybdate is reduced [5].

An assay, using rhodamine B to precipitate phosphomolybdate, was reported recently [6] which covers the same range as the assay of Lanzetta *et al*. However, at phosphate concentrations greater than 1nmol.ml^{-1} the absorbance was greater than 1; for linear measurements, it is advisable to keep the absorbance readings less than 1. Also, rhodamine B is suspected of being a carcinogen and its use therefore requires extra safety precautions.

A variety of compounds have been used to reduce phosphomolybdate. These include ferrous sulphate [7], used in one of the earliest microphosphate assays, hydroquinone [8], 1, 2, 4-aminonaphtholsulphonic acid [9] and ascorbic acid [5,10]. Triton X-100 has been used in place of reducing agents [11] but its major drawback is that the delay between addition of ammonium molybdate and measurement of absorbance must always be identical.

The phosphate released by Na$^+$K$^+$ATPase can be measured in several ways. In NAD-linked assays, hydrolysis of ATP is linked to oxidation of NADH through pyruvate kinase and lactate dehydrogenase [12]. This type of assay is useful for studying the kinetics of ATPase but is unnecessarily complicated for studies where the kinetics are not of primary interest. Use of radiolabelled ATP is a direct but expensive way to assay ATPase activity [13]; measured as the amount of ^{32}P released from ATP labelled on the γ-position. Colorimetric assays have the virtues of being relatively inexpensive and direct. The dye-binding methods are sensitive but have a limited range and require rigorous cleaning of glassware to remove inorganic phosphate.

Of the methods employing reducing agents, the assay of Baginski and Zak [5] is simple to perform, uses relatively low-cost reagents that are easy to prepare and store, and the colour produced is stable for 24 h. In this assay, ascorbic acid is used to reduce phosphomolybdate and free molybdate is complexed with arsenite-citrate reagent so that any inorganic phosphate released after the colour reagents have been added will not affect the results. This technique has been modified for use in low

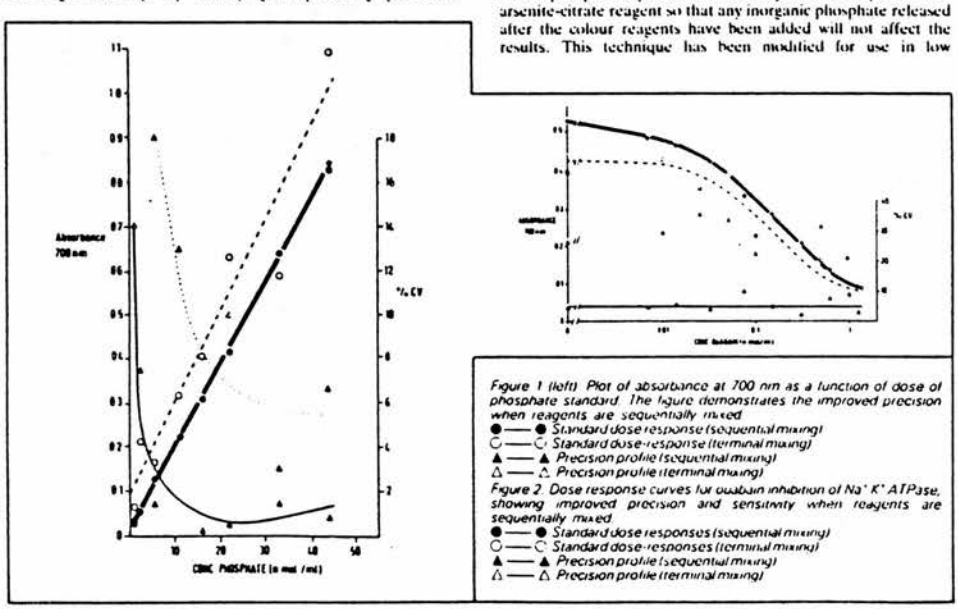

Figure 1 (left) Plot of absorbance at 700 nm as a function of dose of phosphate standard. The figure demonstrates the improved precision when reagents are sequentially mixed
● —— ● Standard dose response (sequential mixing)
○ —— ○ Standard dose-response (terminal mixing)
▲ —— ▲ Precision profile (sequential mixing)
△ —— △ Precision profile (terminal mixing)

Figure 2. Dose response curves for ouabain inhibition of Na$^+$K$^+$ATPase, showing improved precision and sensitivity when reagents are sequentially mixed
● —— ● Standard dose responses (sequential mixing)
○ —— ○ Standard dose-responses (terminal mixing)
▲ —— ▲ Precision profile (sequential mixing)
△ —— △ Precision profile (terminal mixing)

protein-content assays [14] and in assays of microsomal ATPase preparations [15]. Despite the stability of the colour produced, considerable imprecision in early experiments with this method was noted. There were several possible explanations for this lack of precision, and this paper describes their investigation.

Materials and methods

Reagents

All reagents were of analytical grade and obtained from Sigma Chemical Co Ltd, Poole, Dorset, UK.

Phosphate standards (1-88 nmol/ml) were made up by serial dilution of stock standard (0.5 mol/l) with 0.61 M trichloroacetic acid (TCA) and stored at 4°C for eight weeks.

Ascorbic acid (0.57 M) and ammonium molybdate (8.1 mM) were made up in distilled water. Arsenite-citrate reagent contained 77 mmol of sodium-m-arsenite, 34 mmol of trisodium citrate and 10 ml glacial acetic acid made up to a final volume of 500 ml with distilled water.

Imidazole buffer (10 mM), containing sodium chloride (100 mM), potassium chloride (5 mM), magnesium chloride (6 mM) and ethylene glycol-bis (β amino ethyl ether) N,N,N',N'-tetraacetic acid (0.5 mM) was adjusted to pH 7.8 using sodium hydroxide (0.1 M).

Adenosine 5'-triphosphatase (ATPase, 0.75 units/ml, from porcine cerebral cortex) was made up in imidazole buffer. One unit of ATPase activity liberates one μmol inorganic phosphate from ATP per minute at 37°C, at pH 7.8 and in the presence of Na^+, K^+ and Mg^{2+}.

Ouabain standards (0.008-1 nmol/ml) were made up by serial dilution of stock standard (2 mM) with imidazole buffer and stored at 4°C for eight weeks.

All stock standards were stored at −20°C.

Phosphate assay

The assay was based on that of Baginski and Zak [4]. Sample or phosphate standard (50 μl) was added to 1 ml TCA (0.61 M), mixed and left to stand at room temperature for 10 min. After centrifugation (2800 g, 3 min, 4°C), 500 μl of the supernatant was added to 100 μl of ascorbic acid. To this, 250 μl ammonium molybdate were added followed by 500 μl arsenite-citrate reagent. After 15 min the absorbance was read at 700 nm in a Pye Unicam SP8-100 UV/Vis spectrophotometer. Tubes were shaken either after each reagent was added (sequential mixing) or after the final reagent has been added (terminal mixing).

Phosphate generation

100 μl ATP, 750 μl imidazole buffer and 50 μl ouabain standard or buffer were pre-incubated for 10 min at 37°C. The reaction was started by adding 100 μl ATPase and stopped after 60 min at 37°C by addition of 1 ml ice-cold TCA (0.61 M). A sample (50 μl) was taken for phosphate assay.

Results

Figure 1 compares the phosphate standard curves and the precision profile (CV) obtained under conditions of sequential and of terminal mixing of reagents. The equations for these curves are $y = 0.013 + 0.019\,x$ and $y = 0.085 + 0.021\,x$, respectively. The slope of the curve was not altered significantly by sequential mixing of reagents, but precision at each point was considerably improved as compared with the precision observed when terminal mixing was adopted. The mean coefficient of variation (CV) decreased from 18.2% to 4.2% ($p<0.001$); the precision profiles show that the CV on sequential mixing came closer to zero than the CV obtained when the reagents were mixed terminally.

Interassay precision of sequentially mixed assays was measured by triplicate assay of a 44 μmol/l phosphate standard on ten different days over a period of six weeks. The interassay CV was 3.3 ± 0.36%.

Figure 2 compares the precision obtained with the phosphate assay, using sequential and terminal mixing, in a ouabain inhibition study. If the detection limit is defined as the concentration of ouabain which will depress phosphate production by one standard deviation, Figure 2 shows that with sequential mixing the detection limit was improved from 7×10^{-8} mol/l to 4×10^{-9} mol/l ($p<0.001$). This was accompanied by an improvement in mean CV from 28.5 to 5.2% ($p<0.001$).

It was found that a phosphate-independent complex, which absorbs at 700 nm, was formed when addition of arsenite-citrate reagent was delayed. In sequentially mixed assays the absorbance rise due to formation of the phosphate-independent complex did not become significant until the addition of the arsenite-citrate reagent was delayed by 7.5 min. The effect of delayed arsenite-citrate reagent addition was enhanced in phosphate assays in which the reagents were terminally mixed (Table 1).

Discussion

Sequential mixing of phosphate assay reagents produced a marked improvement in precision. This was observed both on a phosphate standard curve (Figure 1) and on a ouabain inhibition curve (Figure 2).

The intercept of the phosphate standard curve decreased as precision improved. The intercept could be due to formation of a phosphate-independent complex, but the high degree of imprecision on the terminally-mixed standard curve could also be responsible for the increased intercept.

Incomplete formation of the phosphomolybdate complex, due to incomplete mixing, is more likely to have been responsible for imprecision. Delayed addition of arsenite-citrate reagent produced a significant increase in absorbance at 2.5 min ($p<0.001$) in terminally mixed assays, but did not produce a significant increase until 7.5 min ($p<0.05$) in sequentially mixed assays. In routine practice, the delay before addition of arsenite-citrate reagent was never longer than 5 min.

Improvement in precision resulted in a considerable improvement in the lower limit of detection of ouabain inhibition of Na^+K^+ATPase. This increases the usefulness of the assay as a means of detecting the low levels of an endogenous, ouabain-like inhibition that is believed to be responsible for the pathogenesis and maintenance of essential hypertension [16]. It is important that the methods used to study this inhibition should be direct, as otherwise the interpretation of these experiments becomes very complex.

Microphosphate assays have a wide variety of potential applications. This assay could also be of value in, for example, the study of myosin ATPase [17] or Na^+K^+ATPase activity of Golgi membranes [18].

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Table 1. *Increase in absorbance as a function of delayed addition of arsenite-citrate reagent in sequentially and terminally mixed phosphate assays.*

	Absorbance at 700 nm as a percentage of time zero value	
Delay time (min)	Sequentially mixed assays	Terminally mixed assays
0	100	100
2.5	99.5	133.1+
5	100.7	133.2+
7.5	103.5*	150.2+
10	102.6*	141.3+
15	107.5+	142.3+
20	113.7+	161.2+

*$p<0.05$ with respect to time zero value
+$p<0.001$ with respect to time zero value

Microcomputer interfacing: an asynchronous serial communications interface

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Abstract
The design and operating details are presented for a low-cost asynchronous serial communications interface for use with Apple II microcomputers. This interface can be operated in RS 232C or 20 mA current-loop modes at switch selectable baud rates. The device permits serial data transfer between computers or between the host computer and suitable laboratory instrumentation.

Introduction
Rapid advances in semiconductor technology have provided laboratory instrumentation manufacturers with a wealth of digital processor devices which can be included in their products. Almost all modern instruments incorporate some form of microprocessor unit and/or some means of interfacing the instrument to a host computer. One common interface is the EIA RS 232C, a bit-serial data transmission standard suitable for low-cost data transfer at relatively low transmission rates. Whilst the full RS 232C standard specifies a 25-pin connector between the computer and the peripheral device, only three interconnecting lines are often required, *viz* transmit data, receive data and a ground line.

A similar interface is the 20 mA current-loop system. This is used in many of the older mechanical typewriters and for applications requiring serial data transmission over extended distances.

Described here is a simple circuit board which performs as an asynchronous serial communications interface which is directly compatible with the Apple II and IIE microcomputer systems and is switch-selectable for operation in RS 232C or 20 mA current-loop modes.

Circuit details
The circuit diagram is illustrated in Figure 1. The interface is designed around the 6850 asynchronous communications interface adaptor (ACIA). The ACIA includes features for formatting and controlling such peripheral devices as modems, CRT terminals and teletype printer/readers. Data transfer between the ACIA and the microprocessor is via eight bi-directional lines that interface directly with the microcomputer's data bus. The direction of data flow (ie receive or transmit) is controlled by the status of the READ/WRITE line from the microcomputer (pin 18 of the Apple edge connector). The primary function of the ACIA is to convert parallel data from the microcomputer bus to serial format and conversely, to convert serial data from the external device to parallel format. Options, such as word length, stop bits and parity checking status are established by writing the appropriate bit pattern into the ACIA's control register.

The circuit also contains a baud-rate generator and selector switches and the necessary line-driver/receiver components to achieve the ±12 V pulses normally used in the RS 232C standard. It can be seen from Figure 1 that the transmit and receive baud-rate lines are connected together, and are switch selectable to provide the usual data transfer rates in the range 50 baud to 9600 baud. The input/output of the ACIA can be switched from the RS 232C line driver/receiver units to the receiver and transmitter of the 20 mA current-loop system.

Operation and software
The Apple II and IIE microcomputers are constructed with edge connector slots for peripheral board expansion. The serial communications interface described here is directly compatible with this arrangement and requires no external power supply unit.

Figure 2 illustrates a typical 'auto-loopback' configuration using a three-wire serial link between the host Apple computer and a peripheral device. Such an arrangement has been discussed by Lesea and Zaks [1] and used for the control of an infra-red spectrometer and for communications between microcomputers and other digital systems, including inter-computer links [2 and 3].

Table 1 provides a simple machine code routine which allows the Apple microcomputer to serve as a 'dumb' terminal for general purpose interfacing, say, to a second computer system. On calling this routine, a master reset of the ACIA is performed and the ACIA is then set for a data standard of eight data bits, no parity check and one stop bit. Further details of setting the ACIA

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Direct demonstration of an inhibitor of sodium, potassium dependent adenosine triphosphatase (Na^+,K^+-ATPase) in plasma from normotensive and hypertensive subjects

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Key words: Hypertension; Na^+,K^+-ATPase inhibitor

Summary

An endogenous inhibitor of Na^+,K^+-ATPase was extracted from human plasma and sera and concentrated by a novel reverse-phase octadecylsilane chromatography method. The active extracts (eluates) were dried and reconstituted in the minimum volume of the non-adsorbed fraction of the plasma from which they had been derived. Reconstituted eluates, non-adsorbed plasma fractions and native plasma samples were then tested for their ability to inhibit phosphate production in standard Na^+,K^+-ATPase incubation mixtures. In a pilot study 31 samples of pooled normal human sera were assayed. The eluates gave a significantly lower production of phosphate than the non-adsorbed fractions or the native sera ($n = 31$, $p < 0.0025$). Further concentration of the eluates by repeated chromatography increased the inhibitory power of the eluate proportional to the concentration achieved, as quantified by ouabain dose-equivalents.

In clinical studies, samples from 12 normotensive subjects and from 12 untreated patients with essential hypertension were tested. Significant inhibition of the ATPase by the eluates, as compared to the corresponding non-adsorbed fractions was seen for samples from both normotensive ($p < 0.05$) and hypertensive ($p < 0.05$) subjects. There was no significant difference in incidence or degree of inhibition between the normotensive and hypertensive groups. This study provides direct evidence for the presence of an endogenous inhibitor of Na^+,K^+-ATPase in human plasma.

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Introduction

High blood pressure is a common condition associated with increased risk of myocardial infarction, cerebrovascular accident (stroke) and renal damage. In over 90% of cases the cause of the high blood pressure is not known ('essential' hypertension), although it may be amenable to pharmacological control with concomitant decrease in the risk of organ damage [1–4].

It has been proposed by de Wardener and MacGregor [5] that the pathogenesis and maintenance of high blood pressure is due to an endogenous inhibitor of sodium transport ('natriuretic factor') secreted in response to dietary sodium-loading in people genetically predisposed to high blood pressure. This inhibitor is believed to act by blocking the ouabain-sensitive component of Na^+,K^+-ATPase; one result of this is increased natriuresis by the kidney. Other side-effects of this inhibitor's action are thought to be increases in intracellular sodium and calcium which, in smooth muscle cells of the blood vessels, lead to an increase in peripheral resistance and, thereby, blood pressure.

Ideally the demonstration of the presence of an inhibitor of Na^+,K^+-ATPase should attempt to address the following points: measurement of inhibition of phosphate production by the ATPase should be direct, the plasma must be extracted with the minimum of insult to its native components, the procedure should allow investigation of a large number of samples in a single assay, utilise samples obtainable from routine clinical practice, and finally the extract should be suspended in a matrix as close to human plasma as possible to prevent unnecessary artefacts. In this study we considered these factors by using a new extraction procedure to concentrate the inhibitor which was then dried and reconstituted in a minimum volume of the non-adsorbed plasma from which it had been extracted. In the pilot study each extract was assayed in parallel with the native serum and non-adsorbed fraction as internal controls; in the clinical study non-adsorbed plasma fractions were used as internal controls. Phosphate production by ATPase was measured directly. We report findings of this pilot study and clinical study using age-matched normotensive and untreated hypertensive subjects.

Material and methods

Reagents

ATP: Disodium adenosine triphosphate (calcium and vanadium free) prepared from equine muscle (Sigma Chemical Co. Ltd., Poole, Dorset, UK) was dissolved in imidazole buffer (vide infra), the pH adjusted to 7.8 with 0.1 mol/l sodium hydroxide, and stored at $-20°C$ in a concentration of 100 mmol/l.

Na^+,K^+-ATPase: Adenosine 5′-triphosphatase (ouabain-sensitive) obtained from porcine cerebral cortex, (Sigma Chemical Co. Ltd.) was stored at $-20°C$ in imidazole buffer (0.75 U/ml). One unit liberated one micromole of inorganic phosphate from ATP per min at 37°C, at pH 7.8 in the presence of sodium, potassium and magnesium ions and excess ATP.

Imidazole buffer: This was 10 mmol/l and contained 100 mmol of sodium chloride, 5 mmol potassium chloride, 6 mmol magnesium chloride (as hexahydrate)

and 0.5 mmol ethylene-glycol-bis(β-aminoethylether) *N,N,N′,N′*-tetraacetic acid (EGTA) per litre. The pH was adjusted to 7.8 with 0.1 mol/l sodium hydroxide.

Arsenite-citrate reagent: This contained 17 mmol sodium-m-arsenite, 34 mmol trisodium citrate and 10 ml glacial acetic acid made up to a final volume of 500 ml with distilled water.

Standards: Working phosphate standards were made up by serial dilution of a 0.5 mol/l primary phosphate standard in 0.61 mol/l trichloroacetic acid (TCA). The working standards were stable for up to 8 weeks at 4°C and covered a range from 1 to 88 μmol/l. The primary standard was stored in aliquots at −20°C.

Working ouabain standards were made over a range of 8 pmol/ml to 1 nmol/ml by dilution in imidazole buffer of a primary (2 μmol/ml) standard. The primary standard was stored in aliquots at −20°C the working standards were stored for up to 8 weeks at 4°C.

All reagents were of analytical grade and obtained from the Sigma Chemical Co. Ltd., St. Louis, MO, USA.

Phosphate assay

Phosphate was measured by a technique based upon that of Baginski and Zak [6]. 50 μl of sample or standard were added to 1 ml of 0.61 mol/l TCA, mixed thoroughly and allowed to stand at room temperature for 10 min, and then centrifuged at 2800 × *g* at 40°C for 3 min. 500 μl supernatant were then added to 100 μl 0.57 mol/l ascorbic acid and vortex mixed. To this mixture was added 250 μl of 8.1 mmol/l ammonium molybdate and the tubes re-mixed by vortex. Finally, 500 μl arsenite-citrate reagent were added, the contents mixed and allowed to stabilise at room temperature for 15 min. The solutions were transferred to microcuvettes (1 ml capacity) and the absorbance at 700 nm recorded on a Pye-Unicam SP8-100 spectrophotometer, using distilled water as blank. Thorough mixing of reagents at each stage is critical to precision [7].

ATPase incubation

750 μl imidazole buffer, 100 μl ATP (50 μmol) and 50 μl sample or buffer were preincubated for 10 min at 37°C before the reaction was started by addition of 100 μl ATPase (0.075 U). The reaction was allowed to proceed for 1 h at 37°C and was then terminated by addition of 1 ml of 0.61 mol/l ice-cold TCA. 50 μl portions of the incubate were then removed for phosphate assay. All assays were performed in triplicate and blanks for substrate, enzyme, sample and incubation time routinely included to correct for endogenous phosphate and enzyme-independent breakdown of ATP.

A standard curve, using known concentrations of ouabain instead of plasma extract samples, was constructed in order to quantify any inhibition by the extracts in terms of ouabain dose-equivalents.

Patients and samples

For the pilot study 31 × 10 ml portions of a pooled human serum which had been stored at −20°C were used. For the clinical study 10 ml blood samples were taken

from the antecubital vein of 12 untreated, newly diagnosed essential hypertensive patients, defined by the World Health Organisation gradings as I or II (MAP 137.3 ± 6.4 mmHg), and 12 age-matched normotensive volunteers (MAP 96.4 ± 2.0 mmHg). Mean arterial blood pressure (MAP) was defined as diastolic blood pressure plus one third of the pulse (systolic–diastolic) pressure. Figures are given ± one standard error of the mean (SEM). The blood was placed in lithium-heparin tubes, centrifuged and the plasma stored at −20°C until required. Re-centrifugation upon thawing was sometimes necessary to remove precipitates induced by freezing.

Extraction procedures

The extracts were prepared from the serum or plasma by reverse-phase octadecylsilane column chromatography (Sep-pak C18, Waters Ltd., Cheshire). The columns were primed with 5 ml of methanol followed by 5 ml of distilled water. The serum or plasma samples (2.5–10 ml as available) were applied to the columns by hand pressure through a nylon 10 ml syringe. The liquid which passed through the column (the non-adsorbed fraction) was retained, as was a sample of the native serum in the pilot study. The column was then washed with 5 ml distilled water and adsorbed material (the eluate) removed from the column with 5 ml of methanol. The eluate was collected in a glass quickfit tube and reduced to dryness under a stream of oxygen-free nitrogen in a water bath at 50°C.

The eluate was reconstituted by adding a minimum volume (350 μl) of its corresponding non-adsorbed fraction and vortex-mixing the tube thoroughly. Eluates, non-adsorbed fractions and native sera were then tested for the ability to inhibit phosphate production in the ATPase incubates. Results from the pilot study indicated that assay of native plasma gave virtually identical results to assay of non-adsorbed fractions. Therefore, for the clinical plasma samples, where volume was critical, the whole sample was applied to the column for extraction.

Statistical methods

Differences between phosphate production in non-adsorbed fractions and eluates were assessed by Student's *t*-tests (paired or unpaired, as appropriate). Probabilities > 0.5 were regarded as not statistically significant. Standard curves were assessed by estimation of the coefficient of variation of the response variable of each standard point ('precision profile'), and linear regression analysis where appropriate.

Results

The phosphate standard curve showed a mean coefficient of variation (CV) of 1% for phosphate concentrations > 5 nmol/ml. Below this precision decreases markedly. In practice, the range of the line used for interpolation was between 15 and 40 nmol/ml. Linear regression analysis with phosphate concentration the independent variable yielded $y = 0.013 + 0.019x$, $r = 1.000$, $p < 0.001$, $sy \cdot x = 0.008$. Interassay precision was assessed by repeated assay of a 44 nmol/ml phosphate standard on 10 different days. The CV was 3.3%. Phosphate production by the ATPase incubates

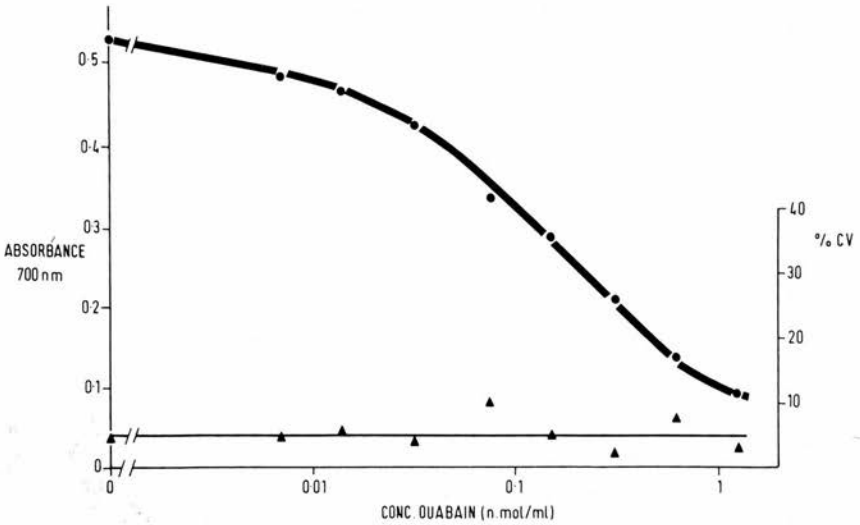

Fig. 1. Ouabain inhibition of phosphate production. Example of a standard curve (●) and precision profile (▲).

was linear with respect to time, up to at least 100 minutes ($y = 0.08 + 0.007x$, $r = 0.995$, $p < 0.001$, $sy \cdot x = 0.024$). The CV of the ATPase incubation procedure as measured on the ouabain inhibition curve was 5.2% (Fig. 1). The detection limit, defined as the dose of ouabain required to reduce the production of phosphate (A700) by one standard deviation, was 4×10^{-9} mol/l.

Since blood samples were collected into lithium-heparinate and the lithium ions remain in the plasma fraction after centrifugation, the effect of lithium on phos-

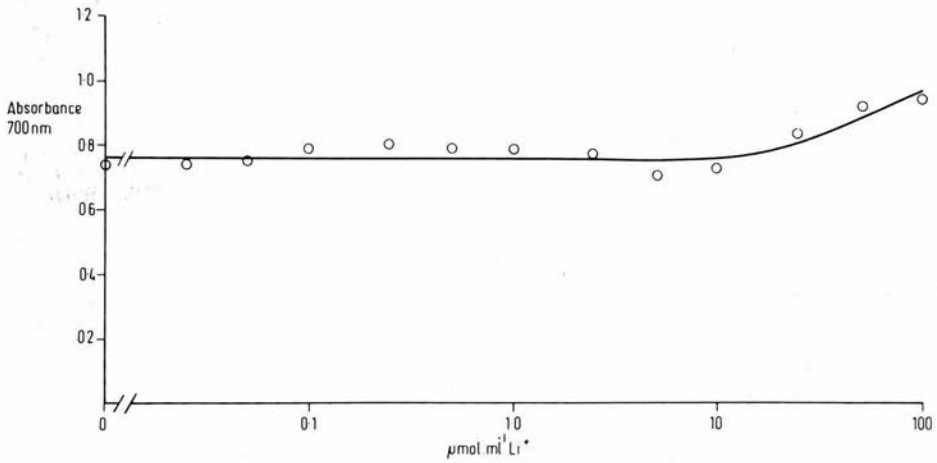

Fig. 2. The effect of lithium ions on the activity of Na^+,K^+-ATPase.

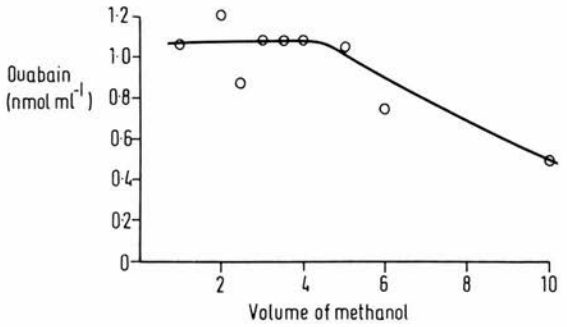

Fig. 3. Recovery of ouabain as a function of methanol volume.

phate generation was investigated. Lithium concentration less than 10 mmol/l had no significant effect, above this concentration slight activation occurs (Fig. 2). When samples are subjected to chromatography virtually all Li^+ remains in the non-adsorbed fraction (mean concentration 0.1 mmol/l). Any small traces remaining on the columns, even if totally eluted in methanol subsequently and concentrated by the procedure described, could not increase Li^+ concentration to greater than 0.35 mol/l in the eluate.

The extraction procedure was tested using plasma samples to which known concentrations of ouabain had been added. These samples were then used as models to assess the adsorption, elution, recovery and reproducibility of the technique. In our hands, a 10 nmol dose of ouabain was all retained by the column whether contained in 2.5, 5 or 10 ml of plasma. No ouabain was lost in the water wash. Recovery of ouabain in the methanolic eluate was around 90% using methanol volumes of 1–5 ml but decreased markedly at higher volumes (Fig. 3).

In the pilot study, comparison of the phosphate production by the untreated sera and the non-adsorbed fractions showed no significant difference (unpaired t-test, $n = 31$, not significant). Comparison of non-adsorbed fractions with eluates, however, showed a statistically significant decrease in phosphate production by the ATPase incubates which contained portions of eluate. ($n = 31$, $p < 0.0025$, mean ouabain dose-equivalent 7.8×10^{-9} mol/l of serum). Further concentration of the eluate remnants by a repeated chromatography step on a fresh column (concentration factor achieved = 11.8) produced, on elution with methanol, a fraction containing 9.0×10^{-8} mol/l ouabain dose-equivalent which compares closely with a predicted value of 9.2×10^{-8} mol/l. The non-adsorbed fraction from this second chromatography step showed no inhibitory capacity.

In the study with samples from normotensive volunteers and untreated patients with essential hypertension there were falls in phosphate production in ATPase incubates containing eluates of these plasmas which, despite the small number of samples, achieved statistical significance (paired t-test) with respect to non-adsorbed fractions in both normals ($n = 12$, $p < 0.05$) and hypertensives ($n = 12$, $p < 0.05$). The results are summarised in Table I. As individual sample sizes were smaller, it was not possible to concentrate the eluates to the same extent as the pilot study and

TABLE I

Inhibition of phosphate production by plasma eluates

The results show that the enzyme activity is significantly reduced in plasma eluates by comparison with non-adsorbed fractions.

	n	$[PO_4]$ produced (nmol/l)	Enzyme activity U/l	Ouabain 'dose-equivalents' (nmol/l)
(a) Pilot study				
Native sera	1	2.92×10^{-5}	55	$<4\times10^{-9}$
Non-adsorbed fractions	31	2.96×10^{-5}	56	$<4\times10^{-9}$
Eluates	31	2.72×10^{-5}	51 *	7.8×10^{-9}
Re-concentrated eluates	1	1.93×10^{-5}	36	9.0×10^{-8}
(b) Normotensives				
Non-adsorbed fractions	12	2.63×10^{-5}	50	$<4\times10^{-9}$
Eluates	12	2.50×10^{-5}**	47 **	$<4\times10^{-9}$
(c) Hypertensives				
Non-adsorbed fractions	12	3.06×10^{-5}	58	$<4\times10^{-9}$
Eluates	12	2.92×10^{-5}**	55 **	$<4\times10^{-9}$

* $p < 0.0025$ with respect to non-adsorbed fractions (unpaired *t*-tests).
** $p < 0.05$ with respect to non-adsorbed fractions (paired *t*-tests).

leave sufficient eluate after assay for re-extraction. When the normotensives and hypertensives were compared with each other (independent *t*-test) no significant difference in incidence or degree of inhibition of phosphate production by the presence of plasma eluate was demonstrable ($n = 12$, not significant). Of the 24 sample eluates, 3 achieved greater than 10% inhibition, 6 greater than 5%; equivalent to ouabain dose-equivalents of 14 nmol/l and 7.2 nmol/l respectively. There was no significant correlation (linear regression analysis) between mean arterial blood pressure and degree of inhibition for either group.

Discussion

Although the phosphate assay worked extremely reliably with a CV of 1%, the ATPase incubation was not ideal because the sensitivity of the method (limit of detectability) was still in the μmol/l range. Thus quantification of the inhibition was subject to considerable imprecision as the change in the response variable at low levels of inhibition is small. The inhibition curves were, however, reproducible and precise and further optimisation may be possible by reduction of ATPase concentration in the incubates. The sample preparation, although not able to concentrate manageable sample sizes sufficiently to overcome the sensitivity problem of the ouabain inhibition curve, did have certain advantages over other techniques. The method enabled a marked degree of concentration (10-fold) with minimal insult to the native plasma or sera and also provided, by means of the non-adsorbed plasma fractions, a matrix for reconstitution which was extremely

close to the original medium. Furthermore, the paired nature of the analysis (eluate versus non-adsorbed fraction) gave a valuable internal control against artefacts, such as matrix effects reported in digoxin immunoassays, and variations in ion concentrations which may affect Na^+,K^+-ATPase activity. The use of blood samples collected into tubes containing lithium-heparinate appears justified (Fig. 2), as the Li^+ ion cannot be selectively concentrated by this chromatography method to a point where it can influence Na^+,K^+-ATPase activity [8]. The use of ouabain-treated plasma as a model to test the extraction procedure indicates that adsorption and elution are highly efficient over a wide range of concentrations and elution volumes. This data does presuppose, however, that the behaviour of ouabain is a satisfactory model for extraction of the endogenous inhibitor. Previous work in this field indicates that the Na^+,K^+-ATPase inhibitor does possess certain ouabain-like properties [9,10]. Similarity of function in terms of ATPase inhibition is not direct evidence for structural homology, but, in the absence of detailed information on the structure of the endogenous inhibitor, it is the closest approximation available. The fall in apparent recovery with large eluant volumes of methanol appears paradoxical, but is most likely to be due to a failure to reconstitute completely the eluate in a small volume of the non-adsorbed fraction, as it must be assumed some eluate dries on the sides of the tube before the evaporation process is completed.

The data from the pilot study provided good preliminary evidence for there being an inhibitor of Na^+,K^+-ATPase present which was concentrated by the chromatography technique. Our findings are consistent with those reported earlier using analogous techniques [11,12]. The data also showed that the non-adsorbed fractions were good internal controls and identical to the native sera in their behaviour in the assay. The further concentration step produced an extract with increased inhibitory capacity to a level directly proportional to the increase in concentration factor. This is evidence for the inhibition being due to a stoichiometric reaction of the Na^+,K^+-ATPase with a specific inhibitor rather than a non-specific effect due to an artefact of the extraction procedure.

Using the extraction technique described in this paper we have concentrated bovine serum, plasma from patients with poly-cythaemia, and plasma pooled from normotensive volunteers. In these concentrated fractions we were able to demonstrate both a dose–response curve for ATPase inhibition as a function of the concentration of the extract, and displacement of radiolabelled digoxin from three different rabbit anti-digoxin sera [13]. These data support the view the endogenous ATPase inhibitor is associated with a cardiac-glycoside-like moiety. The degree of displacement varies markedly, however, between different antisera. This finding highlights the danger of attempting to quantify the apparent concentration of the digoxin-like factor in molar terms.

The inhibitory capacity of the serum eluate was confirmed in the study using plasma samples from normotensive volunteers and patients with untreated essential hypertension. In both groups there was a demonstrable inhibition of phosphate production, although differences in incidence or degree of inhibition could not be established between the two groups, nor could inhibition be correlated as a direct function of mean arterial pressure. Quantification of the inhibition is integrally

dependent upon an inhibition curve of sufficient sensitivity and therefore estimations as 'ouabain-dose-equivalents' must be interpreted with some caution.

If, as these preliminary results indicate, any differences between levels of inhibitor in normotensives and hypertensives are extremely small, it would perhaps reflect the extreme efficiency of such an inhibitor, whereby effective changes in Na^+,K^+-ATPase activity could be mediated via extremely subtle changes in apparent inhibitor concentration or activity.

In conclusion, this study provides a direct demonstration of an endogenous inhibitor of Na^+,K^+-ATPase in human plasma from both normotensive and hypertensive subjects. The technique is simple, reproducible and well-controlled for potential non-specific matrix effects.

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